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AMERICAN VETERINARY MEDICAL ASSOCIATION

## In this issue . . .


- Cyanacethydrazide for Lungworms in Cattle . . 777
- Discontinuity of the Fibula in the Horse . . . . 852
- Renal Function Test in Ewes . . . . . 858
- Brucella Neotomae Infection in Swine . . . . 872
- Infectious Bronchitis Virus Hemagglutination . . 876
- Canine Autogenous Skin Grafting . . . . . 898


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CONTENTS ARE ON PAGES 769, 772, AND 774

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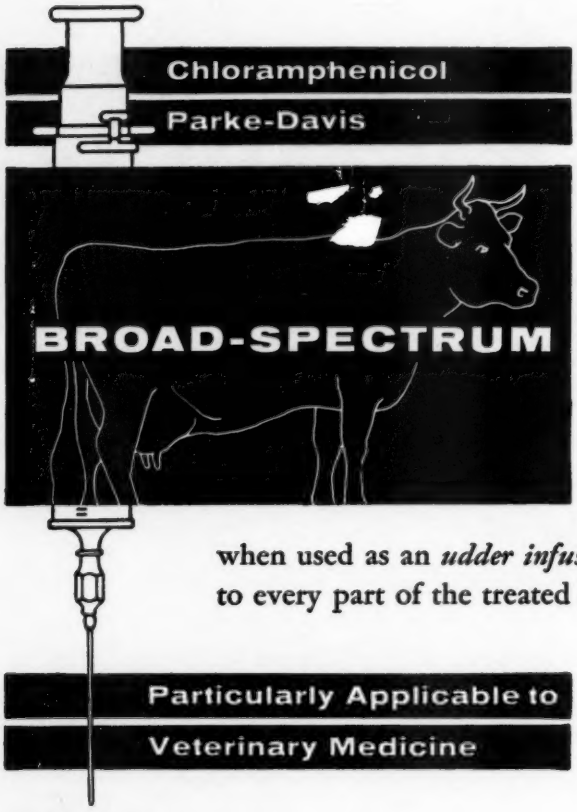


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## Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

**The Efficacy of Cyanacethydrazide as a Treatment of Lungworm, *Dictyocaulus Viviparus* (Bloch), Infections in Cattle** ..... 777
*L. E. Swanson, A. E. Wade, V. F. Senseman, and M. I. Djafar*

**The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as Anthelmintics in Ruminants** ..... 784
*T. J. Galvin, R. R. Bell, and R. D. Turk*

**Studies on *Trichostrongylus Axei* (Cobbold, 1879). IV. Some Aspects of Treatment Pathogenicity, and Quantification in Experimental Infections of a Horse Strain in Calves** ..... 787
*S. E. Leland, J. H. Drudge, Z. N. Wyant, G. W. Elam, and L. B. Hutzler*

***Anaplasma Marginale*. I. Studies with Phase Contrast and Electron Microscopy** ..... 795
*C. Espana, E. M. Espana, and D. Gonzalez*

**The Relationship of Diet to Gastrointestinal Helminth Parasitism in Cattle** ..... 806
*A. Goldberg*

**Multiplication and Cytopathogenic Effect of Foot-and-Mouth Disease Virus in Cultures of Fetal Rabbit Lung Cells** ..... 815
*L. V. Meléndez*

**Experimental Viral Bovine Mastitis** ..... 819
*B. C. Easterday, R. P. Hanson, and J. Simon*

**A Study of the Staphylococci Associated with the Bovine Udder** ..... 825
*W. B. Reid and J. B. Wilson*

**Staphylococcic Phages. I. Lysogenicity in *Staphylococcus Aureus* of Animal Origin** .... 832
*E. H. Coles and A. Eisenstark*

**Staphylococcic Phages. II. The Use of Human Typing Phages and Adpated Human Typing Phages in the Typing of *Staphylococcus Aureus* of Animal Origin** ..... 835
*E. H. Coles and A. Eisenstark*

**Staphylococcic Phages. III. Typing of *Staphylococcus Aureus* Cultures Isolated from the Bovine Udder** ..... 838
*E. H. Coles and A. Eisenstark*

**Histology of the Urethral Epithelium of Domestic Animals** ..... 841
*M. B. Bharadwaj and M. L. Calhoun*

*(Continued on page 772)*

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*(Continued from page 769)*

**A Study of Discontinuity of the Fibula in the Horse**..... 852
*B. Žeškov*

**Phenolsulfonphthalein Clearance as a Measure of Renal Function in Ewes**..... 858
*J. P. Manning, E. F. Reber, O. P. Malhotra, P. D. Beamer, L. E. Boley, and H. W. Norton*

**Ruminant Urolithiasis. I. Preliminary Observations in Experimental Ovine Calculosis**..... 863
*C. E. Cornelius, J. E. Moulton, and B. McGowan, Jr.*

**Experimental Infection of Swine with *Brucella Neotomae***..... 872
*G. A. Beal, R. E. Lewis, N. B. McCullough, and R. M. Claflin*

**Hemagglutination by Trypsin-Modified Infectious Bronchitis Virus**..... 876
*L. J. Corbo and C. H. Cunningham*

**Experimental Ornithosis in Herons and Egrets**..... 884
*R. W. Moore, J. R. Watkins, and J. R. Dixon*

*(Continued on page 774)*

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*(Continued from page 772)*

**A Discussion of Synonymy in Avian and Mammalian Hematological Nomenclature.... 887**
*A. M. Lucas*

**Canine Autogenous Skin Grafting..... 898**
*E. C. Jensen*

**The Narcotic Analgesics and Their Antagonists as Adjuncts to Barbiturate Anesthesia. I. Physiological and Pharmacological Considerations..... 909**
*A. L. Aronson and J. H. Gans*

**Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok Antelope, Using Glycine-2-$C^{14}$..... 917**
*C. E. Cornelius, J. J. Kaneko, and D. C. Benson*

**Studies of Goat Serum Proteins by Paper Electrophoresis..... 921**
*L. R. Gorczyca, R. T. McCarty, J. M. Limon*

**Experimental Brucellosis in White-Tailed Deer..... 925**
*W. G. Youatt and L. D. Fay*

**Scientific Notes..... 927**

**Correction—Lipids in the Epithelium of the Ruminant Forestomach..... 783**

**Classified Advertisement**

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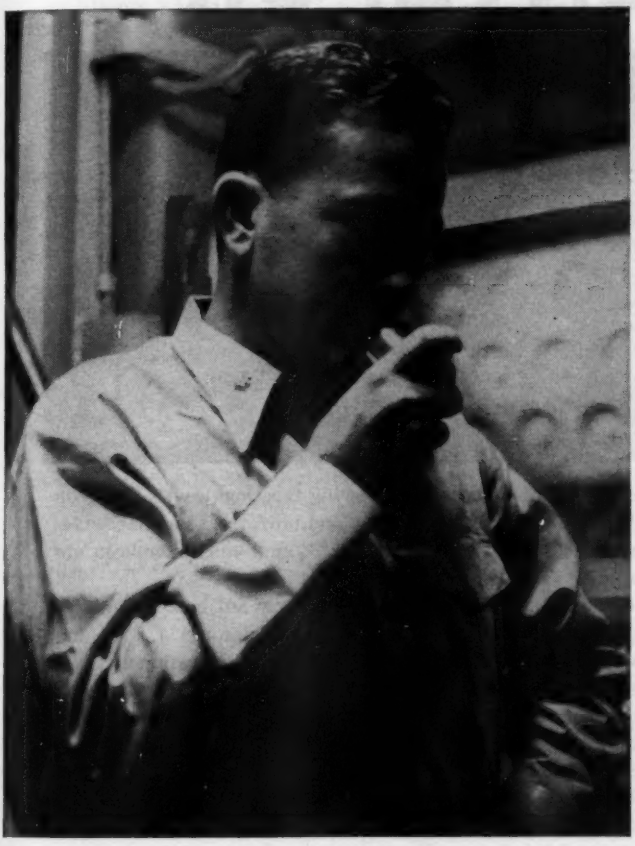

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VOL. XX SEPTEMBER, 1959 No. 78

# The Efficacy of Cyanacethydrazide as a Treatment of Lungworm, *Dictyocaulus Viviparus* (Bloch), Infections in Cattle

L. E. SWANSON, D.V.M.; A. E. WADE, M.S.; V. F. SENSEMAN, B.S.; M. I. DJAFAR, D.V.M., M.S.

*Gainesville, Florida*

LUNGWORM DISEASE of cattle, sometimes called verminous pneumonia or parasitic bronchitis, is caused by the invasion of *Dictyocaulus viviparus* (Bloch) larvae into the lungs and the presence of adults and larvae in the respiratory tract. The disease usually affects cattle 6 months to 1 year old; however, aged cattle may be infected. Death losses may be high but the greatest loss results from poor growth of the affected animals. This disease occurs in many parts of the United States and of the world. It is especially troublesome in Florida. It occurs in other animals such as the American bison, moose, elk, reindeer, and deer which may serve as lungworm reservoirs.

Clinical signs and pathological changes caused by *D. viviparus* infections have been studied extensively.[2,4-7] Most of the chemicals[3,6,7] tested in cattle for their efficacy in removing lungworms have not been effective. Cyanacethydrazide (Dictycide*) has been reported[8,9] as an effective and safe treatment for the expulsion of adult lungworms and as a cure for the disease. Another report[1] has indicated that no distinction could be made clinically between animals treated with cyanacethydrazide and untreated controls.

## MATERIALS AND METHODS

In this study, a sterile solution containing 250 mg. of crystalline cyanacethydrazide per milliliter was prepared just prior to use and injected subcutaneously in doses of 1 ml. per 35 lb. of body weight. A total of 230 cattle were used. Photographs were made of the animals and of the lungs of those in which pure infections were produced in the laboratory. All were killed by gunshot, then the jugular was exposed and severed to permit rapid bleeding but yet prevent inhalation of blood. The entire trachea and each bronchus was cut open and the lungworms were removed and counted. To insure maximum recovery of worms, the lungs were submerged with the cut surface down in a saline solution for 16 hours. This solution was then washed through an 80-mesh screen with fresh saline solution and the residue examined for worms.

*Treatment of Field Infections in the Laboratory.*—Eight calves, 6 to 8 months old, showing clinical signs and harboring heavy infections of lungworms were obtained from a rancher and placed in concrete-floored pens in pairs, one to serve as a control and one (the principal) to be treated. The principals were given one to four injections of cyanacethydrazide subcutaneously at a dose rate of 1 ml. per 35 lb. of body weight, administered at three-day intervals. The calves were slaughtered three to four days after the last treatment. Animals in each pair were handled and treated alike throughout the study. A 20-Gm. fecal specimen was taken each day from each calf, and the lungworm larvae recovered by a Baermann apparatus were counted. The lungs of these calves were examined for gross pathological changes and the lungworms were counted.

From the Department of Veterinary Science, University of Florida, Gainesville. Florida Agricultural Experiment Stations Journal Series, No. 905.

This study was supported in part by a grant from Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

The authors thank Dr. C. F. Simpson, Department of Veterinary Science, University of Florida, for assistance with the pathology and Dr. A. E. Brandt, Section of Statistics, for the statistical analyses.

* The Dictycide used in this study was supplied by Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.



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**TABLE 1—Results of Treatment of Naturally Infected Calves with Cyanacethydrazide**

| Animal No. | Drug injections/intervals* | Larvae averages/20 Gm. feces | | Lungworms† found in lungs | Consolidation of lungs | Fluids in trachea and bronchi |
|---|---|---|---|---|---|---|
| | | Before treatment | After treatment | | | |
| 4 | 3/3 | 486(5) | 15(10) | 0 | extensive | abundant |
| 3 | control | 230(5) | 3(10) | 0 | extensive | abundant** |
| 6 | 4/3 | 941(3) | 139(10) | 0 | extensive | slight |
| 7 | control | 8(3) | 2(10) | 0 | slight | abundant |
| 1 | 2/3 | 3,911(7) | 1,911(6) | 2 | slight | abundant |
| 8 | control | 1,791(8) | 635(7) | 20 | slight | abundant |
| 2 | 1 only | 3,065(10) | 6,460(3) | 18 | slight | abundant |
| 5 | control | 727(10) | 806(3) | 12 | extensive | abundant |

Numbers in parentheses indicate the number of days feces were collected.
* Interval (denominator) between injections is given in days. † Slaughtered 3 or 4 days after last treatment. ** Bloody.

*Treatment of Pure Infections of Lungworms in the Laboratory.*—Twenty-two calves, 3 days old, were placed in dirt-floored pens in pairs and fed a commercial milk replacer from a bottle. The calves had access to fresh water, hay, and a 20 per cent protein commercial calf concentrate. After they were 40 days old, calves were fed a 16 per cent protein commercial concentrate with grass hay and minerals ad libitum. The pens were cleaned twice daily.

Larvae for infecting the initial experimental calves were obtained from field infections. As the study progressed, the control animals were used as sources of larvae. In no case were larvae from treated calves used for the purposes of establishing infections. The first-stage larvae, obtained from the feces of reservoir calves by use of the Baermann apparatus, were allowed to develop to the third stage in Petri dishes containing well water at 17 to 20 C. The numbers of third-stage larvae used to infect calves were estimated by counting representative samples.

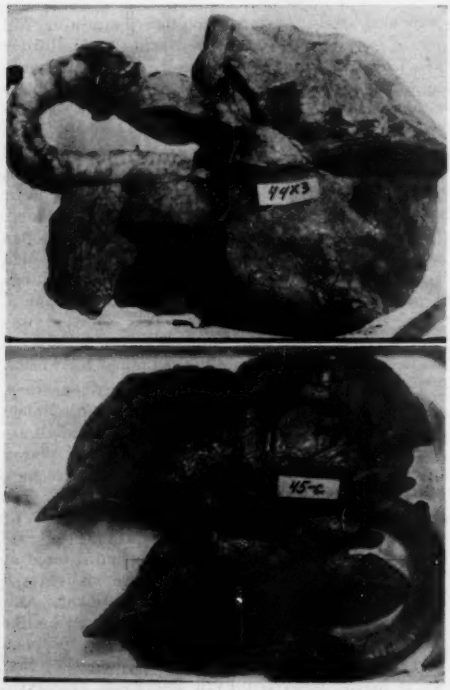


**Fig. 1—Consolidation of the ventral surface of the lungs of calf 44 (treated 3 times) as compared to that of the control calf 45.**

All motile or coiled larvae having a transparent, glistening appearance were classified as living. Slightly curved, opaque larvae containing many undigested food granules were considered to be dead. All experimental infections were produced by the oral administration of third-stage larvae 6 to 30 days old. The calves were infected in pairs by giving each calf 10,000 to 20,000 larvae per day for three consecutive days, resulting in a total dosage of 30,000 to 50,000 larvae per calf. In all cases, each calf in the pair was given the same number of larvae from the same source.

Daily fecal examinations were made from post-infection day 18 until the calves were slaughtered. When first-stage larvae were found in the feces, the calves were moved to concrete pens. The principal in each pair was selected at random and was injected subcutaneously with cyanacethydrazide in doses of 1 ml. per 35 lb. of body weight, on post-infection day 33 to 37 to those given three doses three days apart, and on day 27 or 28 to those given injections for three successive days (table 2). Reactions to the drug were recorded. The animals were slaughtered in pairs from 4 to 40 days after the last treatment. The lungs of these calves were examined for gross pathological changes and the lungworms counted. Sections for microscopic study were made of the lungs of calves 18 to 21.

*Treatment of Field Infections under Field Conditions.*—Two hundred cattle, 1 to 3 years old, were obtained through the cooperation of a Florida rancher for evaluation of cyanacethydrazide under field conditions. Originally, 400 head of thin, weak animals had been removed from a herd of 1,000. They had been treated twice, using a combination of phenothiazine and hexachloroethane at a dose rate of 10 Gm. of each drug per 100 lb. of body weight. Thirty days after the last dose had been administered, 200 of these animals were selected

at random and allotted to two groups of 100 each. The two groups were pastured together and fed supplemental concentrate and minerals during the test. Each group was hot-iron branded for permanent identification and each animal was weighed. Cattle in one group were given cyanacethydrazide, 1 ml. per 35 lb. of body weight, subcutaneously on three successive days. The controls were not handled when the principals were treated.

All survivors were weighed 44 and 87 days later. Necropsies were performed on 20 per cent of the animals that died.

## RESULTS

*Treatment of Field Infections in the Laboratory.*—The results obtained from the administration of cyanacethydrazide to naturally infected calves are summarized (table 1). Although these calves were from the same herd, there is no proof that the original infections were comparable. In all calves except 2 and 5 there was a drop in the number of lungworm larvae recovered from the feces. In the treated calves, no improvement could be determined by clinical signs. The number of lungworms recovered at necropsy and the gross pathological changes of the lungs were similar in the treated and control animals.

Extensive consolidation (involving one or more of the lobes) and slight consolidation (along one or more of the lung borders) each were found in 2 principals and 2 controls. The amounts of fluids found in the respiratory tracts varied and was designated as abundant or slight (tables 1, 2, 5).

*Treatment of Pure Infections in the Laboratory.*—The results obtained from the administration of cyanacethydrazide to experimentally infected calves as compared to control calves are presented (tables 2, 3).

**TABLE 2—Results of Treatment of Experimentally Infected Calves with Cyanacethydrazide**

| Animal No. | Larvae administered | Drug injections/intervals | Average larvae/20 Gm. feces | | Necropsy | | Gross pathology | |
|---|---|---|---|---|---|---|---|---|
| | | | Prior to treatment | After treatment | Days after last treatment | Lungworms found | Consolidation of lungs | Fluids in trachea and bronchi |
| 18 | 50,000 | 3 at 3 day | 8,879(5) | 3,993(10) | 5 | 37 | slight | slight |
| 19 | 50,000 | control | 5,096(6) | 1,507(10) | 5 | 23 | slight | slight |
| 20 | 50,000 | died after 1st treatment | 1,357(4) | died | .. | 1,712 | slight | abundant |
| 21 | 50,000 | 3 at 3 day | 1,408(5) | 404(6) | 5 | 0 | slight | slight |
| 25 | 50,000 | 3 consecutive days | 3,077(4) | 904(6) | 4 | 70 | extensive | slight |
| 24 | 50,000 | control | 24(4) | 41(2) died | 2 | 3,125 | extensive | slight |
| 30 | 50,000 | 3 consecutive days | 1,699(5) | 1,448(4) | 4 | 56 | extensive | abundant |
| 31 | 50,000 | control | 726(5) | 3,207(6) | 4 | 437 | extensive | abundant |
| 40 | 40,000 | 3 consecutive days | 933(3) | 1,395(4) | 4 | 319 | extensive | abundant |
| 41 | 40,000 | control, died day of treatment | ..... | ..... | .. | 2,455 | extensive | abundant |
| 34 | 40,000 | 3 consecutive days | 587(3) | 970(3) | 4 | 305* | extensive | abundant (greenish) |
| 35 | 40,000 | control | 971(3) | 11,787(3) | 4 | 878† | extensive | abundant (greenish) |
| 23 | 40,000 | 3 consecutive days | 6(3) | 168(4) | 4 | 32 | slight | slight |
| 22 | 40,000 | control | 9(2) | 4,966(4) | 4 | 936** | extensive | abundant |
| 26 | 30,000 | 3 consecutive days | 2(2) | 277(41) | 40 | 8 | extensive | abundant |
| 29 | 30,000 | control | 5(1) | 371(41) Last 9 days negative | 40 | 0 | slight | slight |
| 44 | 33,000 | 3 consecutive days | 888(5) | 605(32) Last 3 days negative | 32 | 0 | extensive | abundant |
| 45 | 33,000 | control | 957(6) | 1,039(23) Last 12 days negative | 32 | 0 | slight | abundant |

All larvae were third-stage larvae, administered *per os* in divided doses. Numbers in parentheses indicate days feces were collected.

* 100 were immature lungworms; † 40 were immature lungworms; ** 20 were immature lungworms.

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**TABLE 3—Summarized Results of Treatment of Experimentally Infected Calves**

| Item | No. of animals* | Treatment | Av. No. larvae/20 Gm. feces | | Necropsy | |
|---|---|---|---|---|---|---|
| | | | Before treatment | After treatment | Days after last treatment | Lungworms (av. No.) |
| Control | 1 | control | 5,096(6) | 1,507(10) | 5 | 23 |
| Treated | 2 | 3 injections 3 days apart | 5,144(5) | 2,244(6–10) | 5 | 19 |
| Control | 3 | control | 569(2–5) | 6,653(3–6) | 4–5 | 750 |
| Treated | 5 | 3 successive days | 1,260(3–5) | 977(3–6) | 4–5 | 156 |
| Control | 2 | control | 481(1–6) | 705(32–41) | 32–40 | 0 |
| Treated | 2 | 3 successive days | 445(2–5) | 441(23–41) | 32–40 | 4 |

Numbers in parentheses indicate days feces were collected.
* One treated and 2 control calves died during test and were not included in this table (*See* table 2).

During the course of this experiment it was determined that the administration of a total of 40,000 or 50,000, third-stage larvae was too high, causing deaths, whereas 30,000 to 33,000 larvae established marked infections without death losses. The latter dose was required to establish infections of sufficient degree for adequate evaluation of treatment. The number of adult lungworms recovered from control and reservoir calves at necropsy in relation to larvae given is tabulated (table 4). The average recovery of adult lungworms was 9 per cent of the larvae given to these calves.

Although the data presented (tables 2, 3, 5) may indicate that fewer worms were recovered from the principals than from the controls, the clinical signs and gross pathological changes in the lungs at necropsy were identical in both groups. The areas of consolidation generally were most marked along the borders of the pulmonary lobes; however, the entire lungs were frequently involved.

**TABLE 4—Recovery of Lungworms from Calves in Relation to Number of Third-Stage Larvae Administered Orally**

| Animal | Number infective larvae given | Examined days after infection | Lungworms recovered | | |
|---|---|---|---|---|---|
| | | | Adults | Immature | Percentage† |
| 5* | 70,000 | 19 | 0 | 10,625 | 15 |
| 7* | 70,000 | 28 | 19,255 | 0 | 28 |
| 10* | 70,000 | 29 | 16,032 | 0 | 23 |
| 24 | 50,000 | 29 | 3,125 | 0 | 6 |
| 31 | 50,000 | 34 | 437 | 0 | 1 |
| 22 | 40,000 | 32 | 916 | 20 | 2 |
| 35 | 40,000 | 33 | 838 | 40 | 2 |
| 41 | 40,000 | 22 | 2,455 | 0 | 6 |
| 46 | 30,000 | 35 | 564 | 0 | 2 |

* Reservoir calves. † Average recovery was approximately 9 per cent.

The general gross lesions of the ventral surfaces of the lungs of calf 44, treated on three consecutive days, as compared with those of untreated calf 45 are shown (fig. 1). These calves were held for 32 days fol-

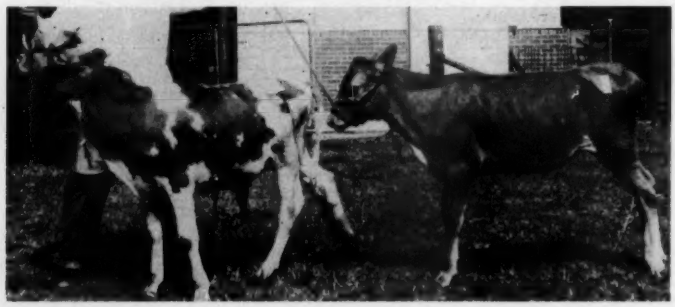

Fig. 2—A pair of calves used in these experiments, 36 treated 3 times (left) and the control calf 43 (right). Notice the trachea tubes (arrows).

lowing the last day of treatment, yet the lungs of the treated calf showed extreme changes involving the diaphragmatic, cardiac, and apical lobes, whereas the control calf's lungs showed few consolidated areas except in the mediastinal lobe. Both calves were free of lungworms at necropsy.

worms were obtained from the controls (table 5).

Approximately 25,000 third-stage lungworm larvae obtained from calves treated with cyanacethydrazide were administered orally to a susceptible calf. The calf died from lungworm infection after 31 days,

**TABLE 5—Lungworms Collected with Tracheal Tubes in Treated and Control Calves**

| Animal | Collections from tracheal tube | | | | Larvae collected/20 Gm. feces | | Gross pathology | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before treatment* (larvae) | After treatment | | | Before treatment | After treatment | Lungworms found | Consolidation of lobes | Fluids in trachea and bronchi |
| | | Larvae | Adults | Immature | | | | | |
| 36(treated) | 3,122 | 13,325 | 2 | 1 | 50(5) | 31(6) | 30 | extensive | slight |
| 43(control) | 200 | 525 | 6 | 0 | 42(5) | 109(6) | 8 | slight | slight |
| 47(treated) | 6,696 | 14,000 | 6 | 0 | 90(6) | 367(6) | 160 | extensive | slight |
| 46(control) | 41,870 | 128,965 | 1 | 0 | 161(5) | 897(6) | 564 | extensive | slight |

Numbers in parentheses indicate days feces collected.
* No adult or immature worms were recovered prior to treatment. All animals were necropsied four days after the last treatment.

Because calf 20 died eight hours after the first injection of cyanacethydrazide, calf 21 was treated and included with calves 18 and 19 (table 2, 3). Microscopic study of lung sections of these 4 calves revealed no differences between control and treated animals.

In order to determine how many lungworms were eliminated from the lungs

and 1,285 lungworms were collected. This indicates that the drug does not destroy the infectivity or markedly alter the virulence of lungworm larvae to calves.

*Treatment of Field Infections Under Field Conditions.*—Under field conditions, the control calves outgained the treated calves (table 6); 64 controls and 60 principals died. There was an average gain per

**TABLE 6—Treatment of Natural Cases in the Field**

| Groups* and No. | Av. weight on 1st day of treatment (lb.) | 44 days | | | 87 days | | | |
|---|---|---|---|---|---|---|---|---|
| | | Death loss† | Average weight (lb.) | Average gain (lb.) | Death loss† | Average weight (lb.) | No. surviving animals | Av. gain of surviving animals during the experiment (lb.) |
| Controls 100 | 298 | 43 | 350 | 31 | 21 | 412 | 36 | 81 |
| Treated 100 | 290 | 41 | 319 | 18 | 19 | 367 | 40 | 54 |

* Duration of infection not known. † 20 per cent necropsied and all had live lungworms.

following treatment, tracheal tubes were inserted into the tracheas of 2 principal and 2 control calves. The tubes served as an effective method of collecting larvae (table 5). There was an increase in larval output in both principal and control calves following the date the principals were treated. All 4 calves had lungworm larvae in their feces, indicating that some had bypassed the tracheal tube. Eight adult and 1 immature lungworms were collected from the treated calves, whereas 7 adult lung-

head of 81 lb. in the control group as compared to 54 lb. in the treated animals. These figures are derived from weights of calves that were in the groups at the beginning of the test and survived throughout the test. Other differences in average weights and gains per head on weighing periods are shown (table 6). Twenty per cent of all animals that died were necropsied, and live lungworms varying from a few to many were found in both treated and control animals. All these animals

XUM

showed varying degrees of pneumonia. Dead liver flukes, *Fasciola hepatica* (Linn), also were found in the livers of all animals necropsied.

## Discussion

The treatment with cyanacethydrazide was of no value in saving the lives or altering the resultant pathological or clinical changes in naturally or experimentally infected animals. The injection caused the animal slight irritation, as evidenced by mild excitement which soon subsided. Although all pairs of calves in the critical tests did not always show the same degree of infection or larval output, the pairs over the whole experiment were comparable (fig. 2).

The results obtained using tracheal tubes and bags did not compare to Wally's [8] experiments, as worms were recovered from the controls as well as the principals. The numbers of lungworms collected were small compared to his, even though the infections were sufficient to cause deaths and produce pathological changes.

Cyanacethydrazide as used in the field herds was of no value and the owner of the cattle was disappointed with the results. Three treatments and handling of the animals on three successive days appeared to be more detrimental to the cattle than were the lungworms. According to the statistical analysis, the survival of 40 and 36 animals in the two groups differently treated shows no significant deviation from what might reasonably have occurred by chance had the two groups of cattle been treated alike. Since this chemical did not prevent death or alter the pathological changes or clinical signs caused by lungworms, it can not be recommended as an effective treatment. Our experiments did not confirm Wally's [8,9] findings.

One group of 5 experimental calves treated (27 to 28 days postinfection) on 3 successive days harbored fewer lungworms than the 3 control calves at necropsy; however, pathologically and clinically they were not different from the controls. This difference in the number of lungworms recovered suggests that cyanacethydrazide may be capable of removing lungworms when used at a particular stage in their development. This might explain the apparent inconsistencies in the results between the various groups of calves used in this study and in other reported experimental findings. The studies involving naturally infected calves where the postinfection dates are unknown indicate that treatment at different stages of the disease does not produce significant reductions in worm burden. In natural infections, lungworm larvae are ingested over relatively long periods and the worms are in various stages of development when the hosts are presented for treatment. Thus, if the drug removes a certain stage only, the effectiveness of the treatment would be unreliable. The treatment may be improved and serve a useful purpose in closely observed gentle dairy calves.

It is known that the injury to the lungs caused by the invasion of lungworm larvae is of prime importance in the pathogenesis of lungworm disease. The first signs of the disease are often observed during the prepatent period, and many animals die of verminous pneumonia before the lungworms reach maturity. However, the mature worms, eggs, and larvae, together with mucus and other fluids, may further aggravate the condition. A therapeutic agent to be of greatest value in this disease must prevent the larvae from reaching the lungs, thereby preventing the resultant pathological changes which occur during the prepatent stages of the disease. In established infections, an effective treatment must virtually remove all worms and promote healing of the diseased part. It must further be remembered that calves recover spontaneously from the disease, a point occasionally overlooked in this type of experimentation.

## Summary and Conclusions

1) This study did not reveal any significant beneficial effect of treating cattle infected with lungworms with cyanacethydrazide (Dictycide).

2) Statistical analysis revealed no significant decrease in larvae output in the treated animals (principals) over the controls.

3) The treatment did not save the lives of animals in the later stages of the disease in the field cases.

4) The treatment did not alter the gross pathology of the lungs of treated animals.

5) Weight gains of animals surviving the 87-day field experiment were in favor of the control calves by an average of 27 pounds per head over the treated animals.

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## Correction—Lipids in the Epithelium of the Ruminant Forestomach

In the article, "The Presence of Lipids in the Epithelium of the Ruminant Forestomach," by Robert E. Habel, in the May, 1959 issue of the *American Journal of Veterinary Research* (p. 437), in the listing of animals under Materials and Methods, the information regarding the last two animals should read "*54—Calf, 15 weeks old.*" and "*55—Calf, 15 weeks old.*"

# The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as Anthelmintics in Ruminants

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RECENT REPORTS in the literature have indicated that certain organic phosphorus compounds have a high degree of anthelmintic activity against gastrointestinal parasites of ruminants.

Herlich and Porter [5] reported that Bayer 21/199 (Co-Ral—0,0-diethyl 0-[3-chloro-4-methyl-7-coumarinyl] phosphorothioate),* given at levels up to 25 mg. per kilogram of body weight to calves and sheep, was relatively nontoxic and removed a large percentage of the worms present. Gordon [2] found that at levels up to 1 Gm./100 lb. of body weight (22 mg./kg.) in sheep it caused some signs of intoxication which resulted in death in a few sheep. At this dosage, it was effective as an anthelmintic.

Radeleff [8] gave Bayer 21/199 to 2 heifers and found that 1 remained normal after being given 25 mg./kg. of body weight while the other died after being given 50 mg./kg.

Gordon [2] also reported on the use of Bayer L 13/59 (Dipterex—0,0-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphonate)* as an anthelmintic in sheep. Dosage levels of 7.5 Gm./100 lb. of body weight (165 mg./kg.) and under were relatively nontoxic to the sheep and lowered the parasite burden as judged by counts of eggs per gram (e.p.g.) of feces. Riek and Keith [9] reported on its efficacy as an anthelmintic in calves. They found that at dosage levels of 5.0 Gm./100 lb. of body weight (110 mg./kg.) the compound was safe and effective in lowering e.p.g. counts.

Levine [6] gave 4 sheep Bayer L 13/59 at rates of 100 to 400 mg./kg. and produced severe signs of intoxication at the highest level. At 200 mg./kg. and above, the drug apparently removed all of the gastrointestinal parasites present.

Radeleff,[7] in checking the oral toxicity of Bayer L 13/59, found that doses of 10 mg./kg. body weight in calves, 100 mg./kg. in cattle, and 200 mg./kg. in sheep caused some signs of intoxication.

A carbamide (Nicarbazin—4,4-dinitrocarbanilide 2-hydroxy-4,6-dimethylpyrimidine),† although not reported to be of value as an anthelmintic, has been recommended as a coccidiostat in poultry. Therefore, a trial was run to determine its efficacy and toxicity. Hammond [3] reported that this carbamide was of little value for the treatment or prevention of coccidiosis in calves.

Because of results obtained by other workers, trials were conducted to determine the efficacy of these preparations, particularly the organic phosphates, as anthelmintics and to determine a dosage level that is nontoxic.

## MATERIALS AND METHODS

Of 15 naturally parasitized calves used in the experiment, 7 were given Bayer 21/199 as a 25 per cent wettable powder in gelatin capsules, 4 were given Bayer L 13/59 as a 50 per cent wettable powder in gelatin capsules, 2 were given the carbamide (Nicarbazin) in water as a drench, and 2 served as controls.

The calves were 5 to 8 months old, had been raised together on pasture for most of their lives, and were in poor flesh. Before treatment, they were placed in a barn on a concrete floor. They were killed eight to 13 days after treatment, with 2 exceptions. One died three and one half days after treatment, and another was held 19 days before it was killed for necropsy.

At necropsy, the abomasum and small intestine of each animal were isolated. The contents of each abomasum and small intestine were washed separately, and an aliquot was taken from each washing by the technique described by Bell.[1] The worms in each aliquot were counted and identified by genus. The total number of each genus in each calf was determined by multiplying by the aliquot factor. After being thoroughly washed, the abomasums and small intestines of 2 calves on each treatment were digested by the method of Herlich [4] and the worms in each sample counted as described.

Another trial was run to check the efficacy of Bayer 21/199 as an anthelmintic in sheep. Fifteen ewes in extremely poor clinical condition were given the compound in a drench. All had lambed during the two weeks prior to treatment, all had been drenched with phenothiazine three to five days previously, and some had been drenched with phenothiazine twice in the two weeks.

## RESULTS

Bayer 21/199 was effective in removing both adult and immature parasites from the abomasums and small intestines of calves (table 1). No signs of intoxication attributable to the drug were seen in calves. Calf 26, which died three and one

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*Available from the Chemagro Corp., New York, N. Y.

† Nicarbazin is produced by Merck and Co., Inc., Rahway, N.J.

half days after dosing, was too weak to stand before treatment.

No Haemonchus spp. and only a few Trichostrongylus spp. were found in the calves that had been treated with this compound. The compound was also effective in removing a high percentage of other genera of parasites, particularly Cooperia spp.

Since 13 of 15 sheep died within a few hours from the effects of Bayer 21/199, parasitological determinations were not made.

Bayer L 13/59 was effective against Haemonchus spp. in calves at the dosage used, and also showed activity against Ostertagia spp. at 200 mg./kg. of body weight. However, this compound was apparently ineffective against the nematodes in the small intestine, particularly Cooperia spp. This was contrary to the results reported by Riek and Keith.[9]

The carbamide appeared to be totally ineffective in removing parasites.

*Bayer 21/199.*—Six of the 7 calves given Bayer 21/199 exhibited no observable signs of intoxication. The death of calf 26, three and one half days after treatment, was considered primarily due to the effects of parasitism.

Different results were obtained from the sheep treated with Bayer 21/199. In the first trial, 14 ewes were treated with 25 mg./kg. of body weight and 1 ewe with 42 mg./kg. After six and one half hours, 3 ewes were salivating excessively and breathing rapidly, while the remaining 12 appeared normal. After 16 hours, 8 ewes were dead and the other 7 showed varying signs of intoxication. Although atropine was administered several times, deaths continued until 72 hours after dosing, when only 2 ewes were alive. One of these had been given the 42-mg./kg. dose.

Since these ewes had all been drenched with phenothiazine three to five days before being drenched with Bayer 21/199, another experiment was performed to determine whether phenothiazine would increase the toxicity of the latter drug.

Of 9 naturally parasitized lambs, 5 were drenched with 25.0 mg./kg. of Bayer 21/199, and 4 were drenched with 25.0 Gm. of phenothiazine and were to be given 25.0 mg./kg. of Bayer 21/199 four days later. However, the first 5 lambs all died, so the 4 given phenothiazine four days previously were given only 12.5 mg./kg. of the compound. No signs of intoxication were seen in these 4 lambs.

*Bayer L 13/59.*—The Bayer L 13/59 was apparently well tolerated by all calves at the 110-mg./kg. of body weight level and by 1 of the calves at the 220-mg./kg.

**TABLE 1—The Efficacy as Anthelmintics of Bayer 21/199, Bayer L 13/59, and Nicarbazin in Calves**

| Calf No. | Compound | Dosage rate (mg./kg.) | Weight (kg.) | Abomasum | | | | | Small intestine | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | T. spp. | H. spp. | O. spp. | C. spp. | U. L. | T. spp. | C. spp. | N. spp. | B. spp. | U. L. |
| 1 | 21/199 | 25 | 132 a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 90 | 0 |
| 4 | 21/199 | 25 | 77 a | 0 | 0 | 0 | 60 | 0 | 0 | 60 | 180 | 30 | 0 |
| | | | b | 0 | 0 | 120 | 0 | 120 | 0 | 60 | 30 | 0 | 60 |
| 9 | 21/199 | 25 | 77 a | 0 | 0 | 120 | 0 | 0 | 0 | 90 | 0 | 30 | 0 |
| | | | b | 30 | 0 | 360 | 0 | 120 | 0 | 0 | 0 | 30 | 30 |
| 12 | 21/199 | 25 | 91 a | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0 |
| 13 | 21/199 | 25 | 75 a | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 0 | 0 | 0* |
| 23 | 21/199 | 25 | 118 a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 21/199 | 25 | 43 a | 0 | 0 | 30 | 0 | 0 | 0 | 0 | 90 | 0 | 0 |
| 2 | L 13/59 | 110 | 68 a | 0 | 0 | 60 | 0 | 0 | 0 | 1,290 | 90 | 0 | 0 |
| | | | b | 60 | 0 | 210 | 0 | 0 | 0 | 0 | 90 | 0 | 0 |
| 7 | L 13/59 | 110 | 98 a | 90 | 30 | 330 | 30 | 0 | 0 | 5,010 | 0 | 0 | 0 |
| | | | b | 120 | 0 | 840 | 90 | 270 | 0 | 1,830 | 30 | 0 | 30 |
| 24 | L 13/59 | 220 | 114 a | 0 | 0 | 0 | 0 | 0 | 0 | 270 | 30 | 90 | 0 |
| 25 | L 13/59 | 220 | 70 a | 0 | 0 | 60 | 0 | 0 | 0 | 2,850 | 0 | 60 | 0 |
| 6 | Nicarbazin | 548 | 73 a | 180 | 1,500 | 420 | 0 | 0 | 90 | 2,460 | 450 | 30 | 0 |
| | | | b | 330 | 270 | 1,020 | 0 | 450 | 60 | 1,950 | 240 | 0 | 270 |
| 10 | Nicarbazin | 561 | 98 a | 150 | 450 | 90 | 0 | 0 | 0 | 6,030 | 0 | 0 | 0 |
| | | | b | 60 | 60 | 300 | 0 | 60 | 0 | 300 | 0 | 0 | 0 |
| 3 | Control | ..... | 166 a | 84 | 1,008 | 252 | 7,056 | 0 | 47 | 4,663 | 0 | 0 | 0 |
| | | | b | 0 | 150 | 600 | 180 | 270 | 438 | 3,942 | 0 | 0 | 0 |
| 11 | Control | ..... | 125 a | 60 | 4,020 | 720 | 0 | 0 | 30 | 2,670 | 270 | 90 | 0 |
| | | | b | 120 | 1,020 | 360 | 0 | 1,380 | 0 | 4,050 | 90 | 0 | 300 |

a=No. of worms in washings from abomasum and small intestine. b= No. of worms obtained by digestion of abomasum and small intestine. * Calf 13 had 22 Moniezia spp.

T. spp.=Trichostrongylus spp.; H. spp.=Haemonchus spp.; O. spp.=Ostertagia spp.; C. spp.=Cooperia spp.; U.L.=unidentified larvae; N. spp.=Nematodirus spp.; B. spp.=Bunostomum spp.

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dosage level. However, calf 24 showed signs of intoxication. On observation one and one half hours after treatment, this calf was in lateral recumbency and was unable to rise; respiration was normal. Six and one half hours after treatment the calf was apparently normal.

*Carbamide.*—At the dosage given, the carbamide produced signs of intoxication. Calf 10 was unable to stand 24 hours after treatment and was finally killed *in extremis* on the eighth day. Calf 6 was obviously in distress after 24 hours but could still walk when killed eight days after treatment. The appetites of both calves were greatly curtailed and, by the time of death, were practically nil.

## SUMMARY

Two organic phosphates, Bayer 21/199 and Bayer L 13/59, and a carbamide, Nicarbazin, were tested for anthelmintic activity in calves. In sheep, the toxicity of Bayer 21/199 was studied. These tests showed that Bayer 21/199 was effective against parasites in the abomasum and small intestine. Bayer L 13/59 was fairly effective against parasites of the abomasum, but exhibited little activity against intestinal parasites. The carbamide was apparently totally ineffective as an anthelmintic even though used in toxic doses. For sheep, Bayer 21/199 proved to be very toxic at a dosage of 25 mg./kg. of body weight.

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# Studies on *Trichostrongylus Axei* (Cobbold, 1879). IV. Some Aspects of Treatment, Pathogenicity, and Quantification in Experimental Infections of a Horse Strain in Calves

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THE ECONOMIC IMPORTANCE of *Trichostrongylus axei* is evident from existing information on its occurrence in natural heavy infections, its wide geographic distribution, the variety of hosts susceptible to infection with it, and its pathogenicity. Gibson,[3] and Turner and Kates,[15] with pure or nearly pure infections, respectively, provided experimental proof of the pathogenicity in sheep of *T. axei* of ovine and bovine origin. Doran,[2] using the same bovine strain of Turner and Kates,[15] reported on the experimental pathogenicity in calves, 3 to 6 months old. Leland and Drudge [6] recorded the pathological effect of a horse strain in experimental infections of rabbits.

The purpose of the present study was to ascertain with a horse strain of *T. axei* in calves (1) the lethal larval dose; (2) the prepatent period; (3) the male/female ratio; (4) the egg-laying potential of the female worm; (5) the pathological effect on the host; and (6) the consequences of therapeutic treatment with phenothiazine.

## MATERIALS AND METHODS

Male calves (12 Jersey and 2 Holstein-Friesian), 3 to 5 months old at the time of infection and raised under helminth-free conditions, were used in the present study. The details of animal facilities, care, diet, larval cultivation, and infection technique were the same as recorded in an earlier paper.[7] The strain of *T. axei* used was originally obtained from a horse in Kentucky (strain A of Drudge *et al.*, 1955 [4]) and maintained in pure infections since 1953. The previous animal passages of the worm for each group of infected calves in the present study was as follows: horse-rabbit to calf H-3; horse-calf-3 lambs to all calves assigned numbers in the 100's; horse-calf-5 lambs to all calves numbered in the 900's; and horse-lamb-3 rabbits to all calves numbered in the 70's.

Egg counting, egg-laying potential of the female worm, artificial digestion, and worm counting techniques were essentially the same as previously recorded [6] except that: (1) the worms were counted in 5- to 10-cc. replicated volumes comprising at least 0.5 per cent of the total volumes from the contents and digested materials; and (2) each 24-hour egg count was the average of five counts by a modified Stoll technique on five undiluted subsamples of a pipettable suspension of the entire 24-hour fecal collection.

Anthelmintic treatment consisted of therapeutic doses (20 Gm./100 lb. of body weight) commercial grade (N.F. green) phenothiazine in the form of a drench and administered via a Whitlock nozzle. The doses ranged from 34.8 to 44.6 Gm. and were given 24 days (calf 72), 60 days (calf 76), and 32 and 57 days (calf 74) postinfection. Drug efficacy was evaluated in the following ways: (1) reductions in 24-hour egg counts; (2) comparison of percentage development in treated animals with that in untreated animals at the same larval dose; and (3) comparison of percentage development in treated animals with the predicted percentage development for an untreated animal (same dose level) derived from regression calculation [12] of data from the 11 untreated animals.

In this study, "infection" refers to the time at which the third-stage (infective) larvae were administered to the host.

## RESULTS AND DISCUSSION

Utilizing the method of Reed and Muench,[9] the data from 11 untreated calves (3 to 5 months old when infected) showed that the 50 per cent lethal dose ($l.d._{50}$) was 500,000 larvae. Comparing these data with the results obtained with a cattle strain where clinical signs were observed with 750,000 larvae and death with 1,000,000 larvae in 56 days,[2] the present horse strain appears somewhat more pathogenic. With the horse strain, clinical signs were evident with 250,000 larvae and death occurred in 19 days after infection with 1,000,000 larvae. Breed differences between the two studies (Doran [2] used 3- to 6-month-old

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TABLE 1—Worm Counts and Related Data Pertaining to Trichostrongylus Axei Infection in Calves

| Calf No. | Age inoculated (days) | No. of larvae administered | Days from inoculation to necropsy | Prepatent period (days) | Worms recovered | | | | Relation to total larvae administered (%) | Male/female | Eggs/24 hr./female | Days from inoculation to treatment | Efficacy (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Abomasal contents | Abomasal digest | Small intestine contents | Cecum and large intestine | Total | | | | Average* | Predicted† |
| H-3 | 141 | 41,000 | 70 | 23 | 24,200 | 368 | 0 | ..... | 24,568 | 59.9 | 0.94 | ..... | ..... | ..... |
| 135 | 178 | 209,312 | 108 | 21 | 61,200 | 600 | 300 | ..... | 62,100 | 29.7 | 0.78 | ..... | ..... | ..... |
| 137 | 109 | 500,000 | D-34 | 22 | 72,800 | 191,380 | 117 | 1,296 | 265,513 | 53.1 | 0.88 | ..... | ..... | ..... |
| 148 | 98 | 1,002,850 | D-26 | 20 | 80,700 | 237,330 | 10,104 | 1,875 | 330,375 | 32.9 | 0.79 | ..... | ..... | ..... |
| H-100 | 115 | 1,430,000 | D-17 | .... | 114,000 | 119,750 | 813 | 12,500 | 247,063 | 17.3 | 0.77 | ..... | ..... | ..... |
| 136 | 111 | 1,500,000 | D-32 | 22 | 101,240 | 75,075 | 858 | 7,700 | 184,873 | 12.3 | 0.63 | ..... | ..... | ..... |
| 933 | 108 | 250,000 | 76 | 23 | 111,500 | 696 | 0 | ..... | 128,900 | 51.6 | 0.79 | 21.8 | ..... | ..... |
| 934 | 109 | 500,000 | 76 | 23 | 26,250 | 3,200 | 0 | ..... | 29,450 | 5.9 | 0.54 | 39.6 | ..... | ..... |
| 932 | 115 | 1,000,000 | D-19 | .... | 186,000 | 801 | 1,800 | 2,640 | 191,243 | 19.1 | 0.72 | ..... | ..... | ..... |
| 71 | 142 | 500,000 | 66 | 20 | 100,496 | 103,000 | 1,500 | 160 | 205,156 | 41.0 | 0.79 | 14.1 | ..... | ..... |
| 73 | 138 | 500,000 | D-24 | 19 | 285,800 | 6,400 | 3,780 | 390 | 296,370 | 59.3 | 0.81 | ..... | ..... | ..... |
| 72 (treated) | 153 | 500,000 | D-39 | 20 | 30,700 | 68,800 | 0 | 10 | 99,510 | 19.9 | 0.67 | 6.1 | 24 | 50.0 | 50.0 |
| 74 (treated) | 123 | 250,000 | 80 | 23 | 1,000 | 600 | 10 | 0 | 1,610 | 0.6 | 1.68 | 70.7 | 32 | 87.6 | 86.2 |
| 76 (treated) | 124 | 250,000 | 83 | 20 | 1,800 | 20,600 | 17 | 0 | 22,417 | 9.0 | 1.50 | 97.0 | 57 | 98.8 | 98.7 |
| Average | 126.0 | ..... | D-27.3 | 21.3 | ..... | ..... | ..... | ..... | ..... | 34.7** | .77**<br>1.28‡ | 41.6 | ..... | 82.6 | 80.4 |

H = Holstein-Friesian; D = died. * Average per cent take in untreated animals at particular larval dose level minus per cent take in treated animals divided by average per cent take in untreated animals times 100. † Predicted per cent take (from regression calculation) at particular larval dose level minus per cent take in treated animal divided by predicted per cent take times 100. ** Untreated only. ‡ Treated only.

Holstein-Friesian calves) do not appear to be significant, since the 2 Holstein-Friesian calves (H-100 and H-3) of the present study did not show any particular refractivity to the infection when compared with the Jersey calves. Indeed, calf H-100 succumbed to the infection in 17 days, which was the shortest interval between infection and death in this study.

Data pertaining to worm counts are presented (table 1).

The worms recovered from untreated animals at death or when killed in relation to the larvae administered decreased in general as the larval dose was increased. However, for the 11 pairs of values, the coefficient of correlation [12] was −0.590 and was not statistically significant. The worm recovery from untreated calves ranged from 59.9 per cent at 41,000 larvae to 12.3 per cent at 1,500,000 larvae with an average of 34.7 in 11 animals. The low worm recovery (5.9%) from calf 934 appeared to be the result of a "self cure." This interpretation is in keeping with the tendency of the blood determinations to return to within normal limits. The clinical signs, and observations made at necropsy, further indicated that this animal was in the state of recovery. Doran [2] observed, in initial infections with calves, a worm recovery which ranged from 8.6 per cent with the administration of 1,500,000 larvae to 30.0 per cent with 100,000 larvae with an average of 19.2 in 5 animals.

In the 7 calves that died (table 1), the interval between infection and death ranged from 17 to 39 days, with an average of 27.3 days. An average prepatent period of 21.3 days in 12 animals was observed. The male/female worm ratio for the 11 untreated animals averaged 0.77 (77 males per 100 females).

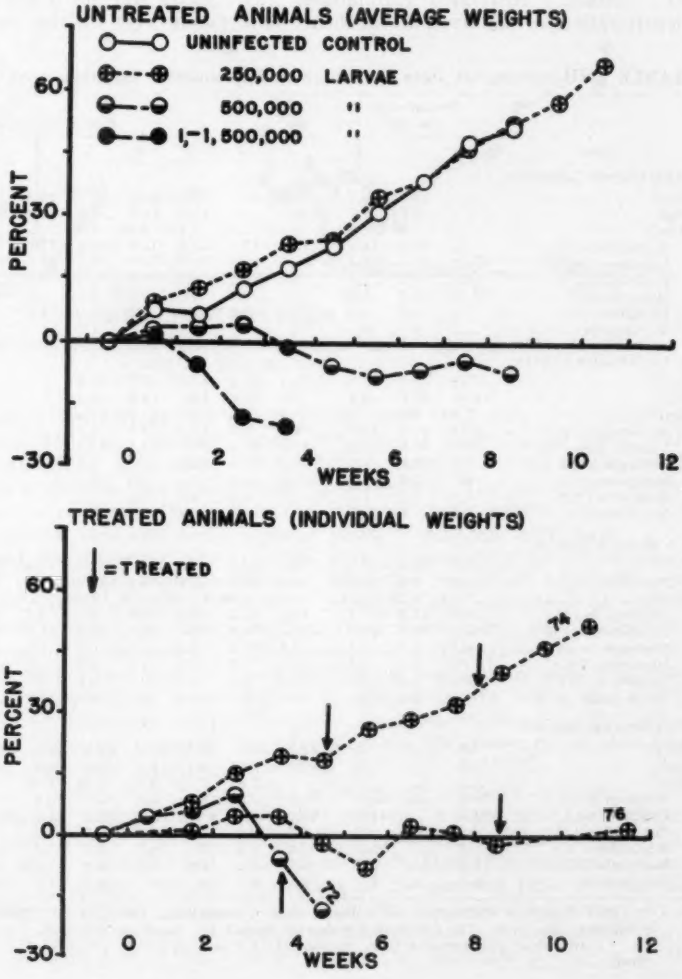
The number of worms recovered from the contents of the cecum and small and large intestines was greater in animals found dead, or necropsied *in extremis*, than in the other animals of the study. This is in keeping with the migration or mechanical translocation of the worm observed in rabbits following death of the host.[6]

The egg-laying potential was evaluated as eggs passed per 24 hours per female and ranged from 6.1 to 97.0, with an average of 41.6 in 6 animals. For the same strain of the worm in rabbits, an average of 20.3 eggs per 24 hours per female was recorded.[6] When the egg-laying potentials of *T. axei* (41.6 eggs/24 hours/female) and Haemon-

chus sp. (5,000 eggs/24 hours/female [5]) are compared, the difficulty in estimating an animal's worm burden from the total egg count in mixed infections becomes obvious.

The influence of infection on the body weight is shown graphically (graph 1) in terms of percentage change from the initial (preinfection) weight. Data averaged for 4 untreated calves at each of the two dose levels (500,000 and 1 to 1.5 million larvae) showed extensive curtailment of weight gain. The weight gain of these calves was well below that of the uninfected control and, for most of the observation period, the body weight was somewhat less than the initial weight. The average weight gain of 2 untreated calves given 200,000 to 250,000 larvae compared favorably with the average gain of 3 uninfected controls. However, in the treated calves (graph 1) given 250,000 larvae the weight gains were, in spite of treatment, less than the control average.

*Pathology.*—Except for treated calf 74, all calves given 209,000 or more larvae showed gross pathological changes at necropsy. The severity of the changes was, in general, proportional to the number of larvae administered. The more consistent




Graph 1—Percentage of change in body weight (lb.) following inoculation with Trichostrongylus axei.

pathological findings were: (1) carcass in extremely poor condition at the higher larval dose levels; (2) little body fat present; (3) degeneration of omental fat; (4) the abomasal mucosa, in acute fatal cases, extremely hyperemic (beet red) with scattered flecks or patches of whitish necrotic debris adhering to the surface (in infections of 8–12 weeks' duration, the mucosa showed a more chronic type response with little hypermia and a swollen and puffy appearance of the mucosa epithelium in the pyloric region); and (5) in severe cases, hyperemia in the first few inches of the duodenum.

Less consistent changes observed were: (1) markedly distended gallbladder, (2) degeneration of fat around the heart and hilus of the kidney; and (3) slight edema of omental fat on greater curvature and pyloric regions of the abomasum. The "ringworm-like" lesions or the progressive growth of tissue in response to the irritative action of the worm observed in the stomach of the horse [10] was not observed in the calves in the present experiments. The duration of infection in these calves may not have been sufficient for changes of his type to occur, although Doran [2] observed lesions similar to the ringworm-like type in calves with infections of comparable duration.

Microscopic examination of sections of the abomasum showed pathological changes which did not differ essentially from those observed in the rabbit.[6] Considerable

**TABLE 2—Hematological Data Pertaining to Experimental Infections with Trichostrongylus Axei**

| Item | Preinfection (weeks) | | | Postinfection (weeks) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 2 | 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| UNINFECTED CONTROLS | | | | | | | | | | | | | | |
| PCV | ..... | 33.9 | 34.7 | 34.6 | 33.9 | 34.1 | 33.0 | 33.0 | 32.8 | 33.2 | 36.2 | 35.0 | 35.6 | 36.6 |
| Hgb. | ..... | 9.7 | 9.6 | 9.9 | 9.8 | 10.0 | 10.0 | 10.2 | 9.2 | 10.0 | 9.8 | 9.4 | 10.2 | ..... |
| RBC | ..... | 7.53 | 7.68 | 8.24 | 7.07 | 7.40 | 8.60 | 8.41 | ..... | ..... | ..... | ..... | ..... | ..... |
| Neutrophils (%) | ..... | 11.2 | 14.0 | 22.2 | 10.5 | 21.5 | 10.5 | 20.2 | 21.0 | 16.5 | 14.0 | 28.5 | 26.0 | ..... |
| Lymphocytes (%) | ..... | 83.0 | 76.5 | 73.5 | 82.5 | 72.8 | 79.0 | 73.7 | 70.5 | 77.5 | 81.0 | 64.5 | 65.0 | ..... |
| Eosinophils (%) | ..... | 0.2 | 2.0 | 0.2 | 0.2 | 0.0 | 0.0 | 1.0 | 0.0 | 0.5 | 2.0 | 1.0 | 1.0 | ..... |
| Monocytes (%) | ..... | 5.0 | 5.5 | 2.2 | 5.5 | 4.0 | 9.0 | 4.0 | 6.5 | 4.5 | 3.0 | 5.5 | 7.0 | ..... |
| Basophils (%) | ..... | 0.5 | 2.0 | 1.8 | 1.2 | 1.8 | 1.5 | 1.0 | 2.0 | 1.0 | 0.0 | 0.5 | 1.0 | ..... |
| No. animals | ..... | 2 | 2 | 2 | 2 | 2 | 2 | 1* | 1 | 1 | 1 | 1 | 1 | 1 |
| 1–1.5 MILLION LARVAE | | | | | | | | | | | | | | |
| PCV | 32.8 | 35.2 | 32.8 | 33.1 | 36.2 | 47.0 | 48.4 | 48.6 | ..... | ..... | ..... | ..... | ..... | ..... |
| Hgb. | 10.0 | 10.2 | 9.4 | 9.8 | 10.9 | 14.0 | 14.4 | 14.2 | ..... | ..... | ..... | ..... | ..... | ..... |
| RBC | 7.84 | 8.00 | 7.58 | 8.24 | 9.61 | 12.1 | 11.51 | 12.2 | ..... | ..... | ..... | ..... | ..... | ..... |
| Neutrophils (%) | 23.0 | 21.0 | 16.6 | 22.3 | 12.8 | 17.3 | 4.8 | 4.0 | ..... | ..... | ..... | ..... | ..... | ..... |
| Lymphocytes (%) | 70.5 | 76.0 | 76.6 | 75.3 | 81.4 | 77.1 | 91.5 | 93.5 | ..... | ..... | ..... | ..... | ..... | ..... |
| Eosinophils (%) | 0.0 | 0.0 | 0.2 | 0.4 | 0.4 | 0.6 | 0.3 | 0.5 | ..... | ..... | ..... | ..... | ..... | ..... |
| Monocytes (%) | 6.5 | 2.0 | 5.4 | 1.1 | 4.3 | 5.0 | 3.3 | 2.0 | ..... | ..... | ..... | ..... | ..... | ..... |
| Basophils (%) | 0.0 | 1.0 | 1.1 | 1.0 | 1.3 | 0.0 | 0.3 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| No. animals | 2 | 2 | 2 | 4 | 4 | 4† | 2** | 1** | ..... | ..... | ..... | ..... | ..... | ..... |
| 0.5 MILLION LARVAE | | | | | | | | | | | | | | |
| PCV | 33.7 | 36.5 | 34.6 | 36.5 | 36.1 | 38.0 | 45.6 | 42.9 | 34.4 | 32.0 | 33.8 | 27.0 | 28.6 | 28.5 |
| Hgb. | 9.8 | 9.8 | 10.3 | 10.4 | 10.7 | 11.0 | 12.4 | 13.2 | 10.0 | 9.8 | 9.6 | 8.2 | 8.5 | ..... |
| RBC | 7.48 | 7.17 | 7.64 | 7.64 | 8.93 | 10.06 | 10.68 | 12.98 | ..... | ..... | ..... | ..... | ..... | ..... |
| Neutrophils (%) | 28.2 | 31.5 | 27.2 | 18.8 | 21.2 | 8.0 | 9.0 | 9.0 | 15.5 | 30.5 | 20.0 | 16.0 | 20.0 | ..... |
| Lymphocytes (%) | 60.8 | 62.5 | 67.0 | 72.8 | 72.2 | 86.5 | 85.8 | 84.0 | 81.0 | 56.0 | 75.0 | 73.0 | 74.0 | ..... |
| Eosinophils (%) | 0.2 | 0.0 | 1.5 | 0.5 | 0.5 | 0.0 | 1.0 | 0.8 | 0.5 | 2.0 | 0.5 | 2.5 | 3.0 | ..... |
| Monocytes (%) | 10.5 | 5.0 | 2.5 | 5.5 | 5.0 | 3.5 | 4.2 | 6.2 | 3.0 | 11.0 | 2.5 | 7.5 | 3.0 | ..... |
| Basophils (%) | 0.2 | 1.0 | 1.5 | 2.5 | 1.0 | 2.0 | 0.0 | 0.0 | 0.0 | 0.5 | 2.0 | 0.5 | 0.0 | ..... |
| No. animals | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2** | 1 | 1 | 1 | 1 | 1 | 1 |
| 0.25 MILLION LARVAE | | | | | | | | | | | | | | |
| PCV | 35.6 | ..... | 37.6 | 34.2 | 34.8 | 36.0 | 34.6 | 35.6 | 33.9 | 36.0 | 36.1 | 36.3 | 38.6 | 39.9 |
| Hgb. | 10.4 | ..... | 11.0 | 10.0 | 9.7 | 10.2 | 9.7 | 10.7 | 10.4 | 10.0 | 10.8 | 11.0 | 11.8 | 12.0 |
| RBC | ..... | ..... | ..... | ..... | 7.82 | 7.35 | 9.50 | 7.48 | 7.72 | 8.37 | 7.09 | 9.08 | 8.96 | 8.88 |
| Neutrophils (%) | 10.5 | ..... | 40.5 | 30.5 | 17.8 | 10.5 | 4.8 | 11.8 | 20.0 | 10.0 | 16.0 | 22.2 | 22.0 | 12.5 |
| Lymphocytes (%) | 81.5 | ..... | 53.5 | 64.0 | 73.8 | 83.5 | 88.8 | 82.5 | 73.5 | 83.5 | 79.5 | 69.5 | 66.8 | 81.0 |
| Eosinophils (%) | 0.5 | ..... | 0.5 | 0.5 | 3.0 | 1.5 | 1.5 | 0.5 | 1.8 | 1.0 | 1.2 | 1.8 | 4.8 | 2.2 |
| Monocytes (%) | 7.5 | ..... | 3.5 | 5.0 | 4.5 | 3.0 | 4.7 | 4.8 | 3.2 | 4.5 | 2.2 | 5.2 | 5.2 | 3.8 |
| Basophils (%) | 0.0 | ..... | 2.0 | 0.0 | 1.0 | 1.5 | 0.2 | 0.2 | 1.5 | 1.0 | 1.0 | 1.2 | 1.2 | 0.5 |
| No. animals | 2 | ..... | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2* | 1 |

PCV = packed erythrocyte cell volume; Hgb. = hemoglobin, Gm./100 ml.; RBC = red blood cell count (millions) per cmm. The differential leukocyte counts are based on 200 cells.

* 1 animal of pair removed from experiment; † 2 animals of the group died; ** 1 animal of the group died.

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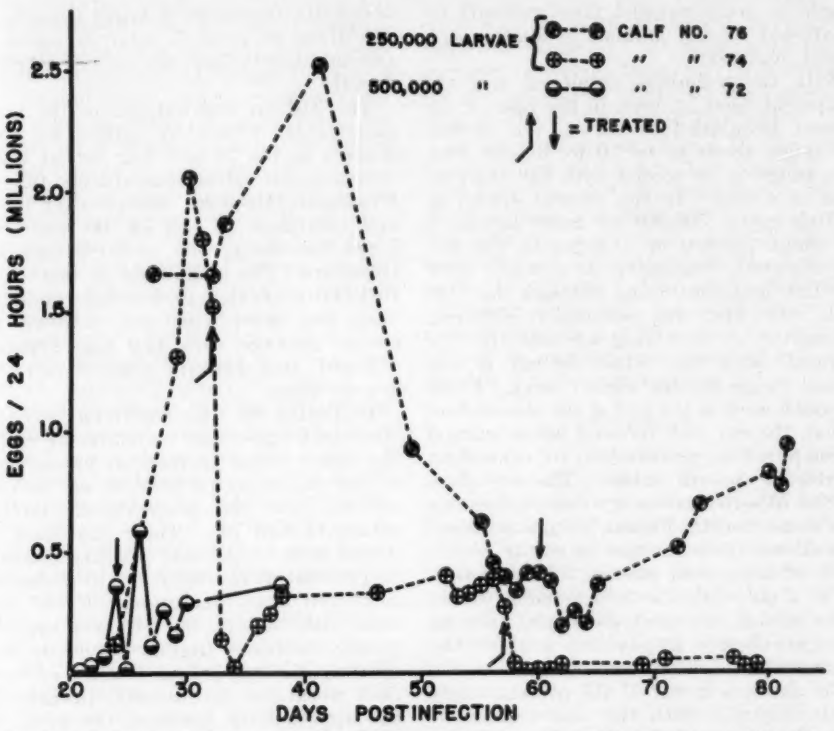


Graph 2—Influence of therapeutic phenothiazine treatment on the 24-hour egg count of Trichostrongylus axei in calves.

sloughing of the surface epithelium was evident. A thick layer of cells, cellular debris, and necrotic material covered areas of the mucosa forming a diphtheritic-type membrane. The majority of worms were within this layer, but worms were also seen in more deeply seated locations in the mucosa. Cells of the lymphoid-macrophage system[14] (lymphocytes predominating) were the principal infiltrating cells, and large numbers were present in the mucosa and occasionally in the submucosa. Eosinophils were rarely seen. Neutrophils were observed in the mucosa of 1 animal (calf 72) when an apparent bacterial invasion of the abomasal mucosa and small intestine occurred.

Occasionally, sections of organs other than the abomasum were made. Usually these were made when the organs did not appear normal grossly. In animals which died, postmortem changes usually were present and complicated interpretation of the observations. Liver sections from 1 animal which died (calf 73), 1 *in extremis* (calf 148), and 1 with clinical signs (calf 934) showed cloudy swelling and intracellular vacuolization. The collecting tubules of the kidneys from calves 73 and 148 showed slight degenerative changes.

Pre- and postinfection blood examinations (table 2), made at approximately weekly intervals and consisting of packed erythrocyte volume (PCV) and hemoglobin determinations and erythrocyte, leukocyte, and differential cell counts, were made for calves numbered in the 100's and 900's. The PCV values were determined for calves numbered in the 70's, but are not shown in the table.

A wide range between maximum and minimal normal values for the cellular components of bovine blood are reported in the literature.[11] It is thus necessary in experiments of the present type to establish the normal for the individual animal by a series of preinfection measurements. Further, during the infection period it is de-

XUM

sirable to make parallel measurements on uninfected control animals of similar age, weight, and sex.

With the technique employed and the equipment used, at least in the case of the present helminth-free calves, the normal PCV range seems to be 30 to 40 per cent. This range is in accord with the observations of Coffin.[1] In the present study, in animals given 500,000 or more larvae, a consistent pattern of changes in the PCV was observed. Beginning three weeks after infection and continuing through the fifth week, the PCV was decidedly elevated. Thereafter, in surviving animals the PCV dropped, with the values falling in the normal range by the eighth week. From the ninth week to the end of the observation period, the PCV was reduced below normal as compared to preinfection or equivalent uninfected control values. The PCV data for the 70 series calves were in conformity with these results. Plasma volume studies[7] have shown these changes in PCV to be the result of a reduced plasma volume resulting in a polycythemic hypovolemia which, if the animal survived, developed into an oligocytythemic hypovolemia after the eighth week.

The changes in PCV of the present study are in contrast with the observations of Doran,[2] who recorded no significant change in hematocrit values, but are consistent with the greater pathogenicity of this horse strain in comparison with his cattle strain. Diarrhea, which was essentially the same as recorded by Doran, no doubt influenced in part the cell to plasma ratio in the early stages of the infection when hemoconcentration was observed.

In essence, the hemoglobin and erythrocyte counts paralleled the changes observed in the PCV.

The differential leukocyte counts indicated an increase in the percentage of lymphocytes from the third through the fifth week postinfection. This observation is in keeping with the large numbers of lymphocytes observed in the abomasal tissue sections. Total leukocyte counts, not shown in the table, remained essentially within normal limits.

*Treatment.*—The small number of treated animals of the present study can not be considered adequate for drawing definite conclusions concerning the efficacy of phenothiazine. However, since the authors are aware of no recorded experience with phenothiazine treatment of heavy experimental infections of pure *T. axei* in calves, the present observations are deemed worthy of report.

The 24-hour egg outputs of the treated animals are presented (graph 2). A reduction in the 24-hour egg output was observed in all calves immediately following treatment. However, except after the second treatment of calf 74, the egg counts began increasing one to three days after treatment. The magnitude of these counts following treatment becomes somewhat more impressive when one considers that, on the average, each 41.6 eggs represents 1 female and perhaps slightly more than 1 male worm.

Following the temporary (one day) posttreatment egg-count reduction of calf 72, the count began increasing, but never attained the expected level of an untreated animal (*i.e.*, the pretreatment levels of calves 74 and 76). These egg-count data would seem to indicate effective treatment, except that at necropsy 99,510 worms were recovered from this animal. From experience with rabbits, the 24-hours egg count usually decreases slightly before death, indicating the egg-laying activity of the female worm may be adversely influenced by the approaching death of the host. This may have been the case in calf 72. A value of 6.1 eggs per female per 24 hours for this animal was the lowest of the 6 animals measured for egg-laying potential of the worm.

In calf 76, the average 24-hour egg count for the three days prior to necropsy was approximately twice the average for the three-day period prior to treatment. Before treatment, a peak in the egg-count curve was observed which may have been due to operation of a self-cure phenomenon, or merely represented the maximum egg-count peak observed in initial experimental infections.[6] At the time of treatment, 60 days postinfection, this calf showed severe signs of clinical parasitism. To what extent worms were spontaneously lost before the treatment was instituted was not evaluated.

The two treatments given 32 and 57 days postinfection to calf 74 resulted in substantial reductions in the egg counts. However, the rebound in the egg count following the first treatment suggested that on the basis of 41.6 eggs per female per 24 hours, some 8,000 females or approximately 16,000 male and female worms were not

removed (efficacy 86.2%). Following the second treatment, the egg-count average for the three-day period prior to necropsy indicated (assuming 41.6 eggs/female/24 hours) some 2,040 worms were not removed. This estimate compares favorably with the 1,610 worms actually recovered from this animal at necropsy.

In critical tests on animals with mixed infections, Swanson *et al.*[13] (at 21–48 Gm./100 lb. in 4 animals) and Porter [8] (at 5–17 Gm./100 lb. in 4 animals) record efficacy for *T. axei* at 100 and at 89.6 to 100 per cent, respectively, with phenothiazine. In the present work, at 20 Gm./100 lb., an efficacy of 50.0 to 98.8 per cent was observed in 3 animals (table 1). Since the worms eliminated in the feces following treatment were not collected, the tests of the present study can not be compared with the previously mentioned critical tests [8, 13] without qualification, *i.e.*, cognizance of the fact that the number of worms existing in the animals prior to treatment was based on the adults recovered from untreated control animals.

Of particular interest is the large number of worms recovered following treatment (99,510 worms, efficacy 50.0% in calf 72; 22,417 worms, efficacy 80.4% in calf 76; 1,610 worms, efficacy 98.7% following two therapeutic treatments in calf 74). The worms remaining in calf 72 plus the cumulative effects of the infection were apparently sufficient to cause death 15 days after treatment. Treatment of this calf shortly after the average prepatent period, when some of the worms had not fully matured and were less susceptible to the drug, may have been a factor in the poor response to treatment. If a large number of immature worms were not removed by treatment, one would expect a rise in the egg count as the remaining worms matured. However, as mentioned above, the marasmatic condition of this host may have been responsible for the low egg count. The worm burden of 22,417 in calf 76 on post-treatment day 23 could conceivably be an adequate number to produce clinical parasitism in calves of this age, considering that untreated calf 934 harbored 29,450 worms and showed marked clinical signs of parasitism. Two treatments were necessary to reduce the burden of calf 74 to 1,610 worms.

The recent use of the artificial digestive procedure for freeing *T. axei* from the wall of the abomasum with resulting higher worm recoveries may indicate that the early critical work with this worm overrated the efficacy of the drug, since larger numbers of worms than were recovered by the methods then employed may have existed in the animals after treatment.

In treated animals 74 and 76, the male/female worm ratio was 1.68 and 1.5, respectively. In calf 72, however, in which the treatment was least effective, the ratio was 0.67. A mean ratio of 0.77 was obtained for 11 untreated animals. A statistically significant (1% level) difference was observed between the mean for the untreated animals and the mean for animals 74 and 76, suggesting a selective action of the drug on the female worm.

## Summary

Data from 11 calves, 3 to 5 months old at the time of infection, indicated that the 50 per cent lethal dose was 500,000 larvae. Pathogenicity of this horse strain of *T. axei* to calves appears greater than that of other strains studied in these animals. The worm recovery from 11 untreated calves ranged from 59.9 per cent at 41,000 larvae administered to 12.3 per cent at 1,500,000 larvae administered, with an average of 34.7 per cent. The interval between infection and death ranged from 17 to 39 days, with an average of 27.3 days. The average prepatent period in 12 calves was 21.3 days. The male/female worm ratio in 11 untreated animals averaged 0.77. The egg-laying potential in six animals ranged from 6.1 to 97.0 and averaged 41.6 eggs per 24 hours per female worm.

The more consistent pathological findings were: (1) body weight was reduced; (2) carcass was in extremely poor condition at the higher larval dose levels; (3) very little body fat was present; (4) degeneration of omental fat was observed; (5) the abomasal mucosa in acute fatal cases was extremely hyperemic (beet red) with scattered flecks or patches of whitish necrotic debris adhering to the surface, while in infections of 8 to 12 weeks' duration it showed a more chronic response with a swollen and puffy appearance of the mucous epithelium in the pyloric region with little hyperemia; and (6) in severe cases the hyperemia was also observed in the first few inches of the duodenum. Microscopic examination of sections of the abomasum revealed a thick layer of cells, cellular debris, and necrotic material covering areas of the mucosa,

XUM

forming a diphtheritic-type membrane, and sloughing of the surface epithelium.

In calves given 500,000 or more larvae, the packed erythrocyte volume (PCV) was decidedly elevated three weeks after infection. Thereafter, in surviving animals the PVC dropped, with values falling in the normal range by the eighth week. From the ninth week to the end of the observation period, the PCV was reduced below normal as compared with preinfection or equivalent uninfected control values.

Differential leukocyte counts indicated an increase in the percentage of lymphocytes from the third through the fifth week postinfection.

The administration of phenothiazine at 20 Gm./100 lb. resulted in a treatment efficacy of 50.0 to 98.8 per cent in heavily infected animals. Two treatments at this dose level were necessary to achieve the 98.8 per cent worm removal. However, in spite of a removal efficacy this high, relatively large numbers of worms were present in this and other treated calves at necropsy. Limited data are suggestive of a selective action by the drug on the female worms.

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# *Anaplasma Marginale*. I. Studies with Phase Contrast and Electron Microscopy

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ALTHOUGH *Anaplasma marginale* has been recognized as the causative agent of an important disease of cattle for nearly 50 years, there is still no unity of opinion as to the exact nature of the parasite. Early workers classified the organism as a Protozoa, in spite of its lack of differentiation into cytoplasm and nucleus and the absence of any evidence of a life cycle. Some investigators are inclined to consider the Anaplasma as bacterial in nature, while others believe it may belong to the Rickettsiales or the Virales. The generally accepted morphology of the agent as observed in blood smears stained with Romanowsky's stains is of little aid in elucidating the nature of the parasite. The Anaplasma appears at the margin of infected erythrocytes as a bluish purple, compact, homogeneous body measuring from 0.2 to 1.0 $\mu$ in diameter and consisting of chromatin material, completely devoid of cytoplasm. However, since 1914, when Quevedo [17] first described elongated forms with fine, flagella-like prolongations, a number of workers have reported similar findings which would point to the protozoan nature of *A. marginale*.

These various studies will not be reviewed here since this was amply done both by Dikmans [7] (1933) and Lotze [16] (1942), who also described elongated forms. The only recent report of the findings of tail-like projections is that of Franklin and Redmond [14] (1958). In all of these investigations the Anaplasma bodies were variously described as having protrusions, as though sprouting; as having a dense spherical portion with a filamentous tail, which might be double; or as appearing like commas, comets, rings, or dumbbells. The demonstration of these forms in most of the studies required special treatment of the erythrocytes, special fixation and staining of smears, and even special illumination for microscopic observation. They were observed only in an occasional erythrocyte and sometimes only at a certain stage of infection.

The most convincing description of such forms and the best photomicrographs of rings and comets appear in a paper by Boynton.[2] He was able to demonstrate these structures either by fixing films of blood with osmic acid vapor or fixing smears prepared 24 to 48 hours before with 1 per cent formalin and then staining with Jenner's and Giemsa's stains or with Toisson's fluid. Boynton stated that the comet forms appeared "when the substance of the cell carrying the hemoglobin is drawn to the bodies in the stroma and gives the appearance of a comet-like tail." He believed that the ring substance "may be composed merely of hemoglobin or may turn out to be a link in the evidence establishing the marginal body as a living organism."

As far as we know, evidence of a possible life cycle in *A. marginale* has been presented by only two investigators. Lotze,[16] on the basis of the study of smears stained with Wright's followed by Giemsa's stain, divided the Anaplasma bodies into two types: bodies which were spherical with smooth contours, measuring from 0.2 to 0.5 $\mu$ in diameter, and those which were roughly spherical or ovoidal (occasionally cuboidal), measuring from about 0.6 to 0.9 $\mu$. In smears of blood first hemolyzed with 0.5 per cent acetic acid, he found that the small bodies retained their integrity while the large ones became swollen, revealing eight small, round "sporoid bodies." The large anaplasmata might rupture, resulting in the dispersion of the small bodies within the cell. In studies with electron microscopy, De Robertis and Epstein [6] showed the Anaplasma to be composed of a central undivided mass and peripheral "elementary bodies" of high electron density, 170 to 220 m$\mu$ in diameter, or of tightly packed submicroscopic bodies. In some cases, they also found a large number of small bodies dispersed throughout the erythrocyte. These findings were in part confirmed by Espana,[9] although he was unable to find large numbers of submicroscopic bodies distributed throughout the stromata.

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A number of investigators[2-5, 7, 8, 12] have carried out filtration studies in an attempt to show that the agent of anaplasmosis is a filterable virus. With the single exception of those of Foote,[12] these experiments have been negative or inconclusive. Foote[11] believes *A. marginale* to be a virus and the Anaplasma as seen in infected erythrocytes to be an inclusion body. According to this worker, electron photomicrographs of ultra-thin sections of infected erythrocytes, showing from one to seven masses of particulate matter in a clear zone, support this view.[10] On the basis of size, the findings of De Robertis and Epstein and Espana could also support the virus theory of the cause of the disease. However, since the submicroscopic bodies might represent a stage in the life cycle of a more complex parasite, the electron microscope studies might suggest, as well, that *A. marginale* is a Protozoa. Thus, it is evident that none of the observations reported to date seems sufficiently convincing for the unequivocal classification of this organism.

The purpose of this work was to study the morphology of *A. marginale* with the phase-contrast microscope using fresh, unstained, infected erythrocytes with the hope of being able to demonstrate structures not visible in fixed, stained preparations. Since phase microscopy revealed forms which had not been seen earlier with electron microscopy, further studies were carried out with the electron microscope using the same preparations which were used for the phase contrast work. Although the studies to be reported are of a preliminary nature and the data are as yet inadequate for making definitive conclusions as to the nature of the parasite, we believe that the observations are sufficiently striking to deserve reporting at this time.

## MATERIALS AND METHODS

*Source of Anaplasmata.*—The study was done with a strain of *A. marginale* isolated from a bovine carrier from the state of Michoacan, Mexico. The parasite has been maintained by passage in intact adults and splenectomized calves. Infected erythrocytes for this study were from a relapsed splenectomized carrier (animal 4); 7 experimentally infected, acutely ill animals (31, 11, 12, 15, 22, 27, and 7, representing various passages of the *A. marginale* originally isolated from carrier animal 4); 1 animal (33) which was infected with *A. marginale* after passage through 5 calves and 1 sheep; and 1 animal with a field case (1F) which represents a different isolate of the organism.

Control observations were made on the cells from 4 normal cattle and from 1 healthy carrier in which no anaplasmata were demonstrable in stained smears. Fresh blood was used in the case of 2 of the 8 experimentally infected cattle, the field case, the carrier, and 3 of the 4 normal animals. Blood from 5 of the 6 remaining experimentally infected animals and from 1 normal animal had been stored at −20 C. for varying periods of time. Blood from the original carrier and the eighth infected animal had been lyophilized and stored at room temperature for three years.

*Collection and Handling of Blood.*—Samples of blood were taken from animals during various stages of the disease when 15 to 60 per cent of the erythrocytes were invaded. The blood was collected in two volumes of Alsever's solution and the cells washed four or five times with physiological saline solution. Fresh blood was stored as a 50 per cent cell suspension in veronal buffer, Hornibrook's,[15] or Bovarnick's[1] solution at 5 C. The cells which were frozen were suspended in water, physiological saline solution with 5 per cent glucose, or Hornibrook's or Bovarnick's solution and stored at −20 C. for from 18 days to two and one half years. The menstruum for the cells which were lyophilized was also Bovarnick's solution.

*Preparation of Hemolysin.*—Specific hemolysin was prepared in rabbits by giving them intravenous injections of 1.0 ml. of a 50 per cent suspension of bovine erythrocytes on three consecutive days. After a rest of four days they were given an intraperitoneal injection of a suspension of erythrocyte stromata, equivalent to 0.5 ml. of packed cells, followed on the next two consecutive days by intravenous doses of twice the previous amount of stromata. A third series of injections, identical to the second, was given after another four-day rest and blood samples were taken nine days after the last injection.

*Preparation of Stromata.*—The erythrocyte stromata were prepared as follows: 1 volume of distilled water was added to 1 volume of washed erythrocytes. Hemolysis was allowed to take place for 30 minutes at room temperature. Eighty volumes of distilled water, saturated with $CO_2$ and cooled to 5 C., were then added and the mixture allowed to stand at 5 C. overnight. The stromata clumped and settled spontaneously after standing. The supernatant fluid was decanted and the sediment washed several times with cold $CO_2$-saturated water, until the supernatant fluid had almost no color. The stromata were then washed with 0.1 per cent NaCl, saturated with $CO_2$, in order to remove as much hemoglobin as possible. The last two washings were carried out with cold distilled water. The stromata which were separated by this method were almost without color. To prepare the antigen for the rabbit immunization, the stromata were measured by volume, the acidity was neutralized with 1.4 per cent $NaHCO_3$ and isotonic buffered saline solution (pH 7.2) added to make a 10 per cent suspension.

*Complement.*—The complement consisted of pooled serum from 10 guinea pigs. It was distributed in ampules, sealed, and stored at −20 C.

*Saponin.*—A stock solution of 5 per cent saponin in physiological saline solution was prepared, filtered through a Seitz filter, and stored at

room temperature. Dilutions were made just before use and fresh stock solutions prepared at least once a week.

*Preparation of Blood for Phase Microscopy.*—Specific Hemolysis.—Equal volumes of a 2 per cent suspension of washed erythrocytes, several dilutions of hemolysin, and complement (2 units) were mixed and observed immediately so that the process of lysis could be followed. The dilution of serum chosen for use was that which produced hemolysis slowly enough so that unhemolyzed cells, as well as cells in various stages of hemolysis, could be observed simultaneously.

Saponin Hemolysis.—Various concentrations of saponin, ranging from 0.04 to 5.0 per cent, were employed. The optimal concentration for hemolysis was determined by mixing washed packed cells or a 50 per cent cell suspension with 20 volumes of the several saponin dilutions and incubating in a 37 C. water bath for 15 minutes. The dilution chosen (usually 0.08 to 0.3%) was, as in the case of hemolysin, that which produced hemolysis slowly. Preparations were observed immediately after mixing the blood cells and saponin so that hemolysis could be followed.

Frozen Blood.—Observations were made with cells which had been stored at −20 C. immediately after thawing. It was found, however, that the stromata were clearer and the anaplasmata better defined if the preparations were allowed to remain at room temperature for several hours before being studied. In some cases, saponin was added to the partially hemolyzed cells to produce complete hemolysis.

Lyophilized Blood.—Samples were reconstituted with sterile distilled water, allowing the cells to rehydrate slowly. Observations were made on the suspensions without further treatment.

All samples were prepared on slides with cover glasses and sealed with paraffin so that they could be observed over a period of several hours.

The microscope used in these studies was a Leitz Ortholux* with a Heine condenser.† The objectives were a Pv Apo L/40/0.70 n, which has a phase ring of normal absorption of 75 ± 5 per cent, and a Pv Fl oil 70/1.15 h with a phase ring of high absorption (88 ± 2%).

*Preparation of Blood for Electron Microscopy.*—Suspensions of infected erythrocytes prepared for phase microscopy were also used for electron microscopy. The lysed cells (hemolysis accomplished either by saponin or specific amboceptor) were centrifuged at 5 C. in a refrigerated centrifuge** at 3,000 r.p.m. for 15 minutes. The supernatant fluid was discarded and the stromata washed twice with 10 volumes of veronal buffer (pH 7.4). After the last washing the sediment was resuspended in sufficient buffer to give a suitable concentration of stromata for observation with the electron microscope.

Grid mounts were made as follows: smears of each suspension were made on plain 1 per cent agar plates, allowed to dry at room temperature, and then covered with collodion. Once dried, the collodion film carrying the cell membranes was floated on distilled water and transferred to nickel grids. A conventional method of preparing the grid mounts was also employed. By means of a micropipette, drops of the stroma suspension were transferred to nickel grids with collodion film supports. In both cases, the films were left to dry at room temperature and examined after light shadowing with palladium at arc tangent 2/10.

A Philips 100 kv. electron microscope ‡ was used in these studies. The magnification generally employed varied from × 5,000 to 20,000.

## RESULTS

*Phase Contrast Microscopy.*—The majority of the observations were made on fresh blood from animal 31 which had an acute, fatal case of anaplasmosis. At the time the blood was taken, 45 per cent of the erythrocytes were infected. Preparations were studied on the same day that the blood was withdrawn and after storage in Bovarnick's solution at 5 C. for one to 40 days and at −20 C. for 18 days. Findings were confirmed with fresh blood from 2 other animals (1 experimentally infected and the other a field case, representing a distinct isolate of *A. marginale*) and with frozen or lyophilized blood from 7 other infected animals (table 1).

**TABLE 1—Description of Erythrocytes Used for Phase Contrast Microscopy**

| Animal No. | No. of passages | Infected erythrocytes (%) | Blood storage | |
|---|---|---|---|---|
| | | | Temp. (C.) | Time |
| 31 | 5 | 45 | 5 | 0–40 days |
| | | | −20 | 18 days |
| 33 | 6* | 60 | 5 | 0–6 days |
| 1F† | 0 | 20 | 5 | 5–12 days |
| 11 | 5 | 23 | −20 | 16 months |
| 12 | 2 | 18 | −20 | 30 months |
| 15 | 6 | 45 | −20 | 16 months |
| 22 | 5 | 20 | −20 | 9 months |
| 27 | 5 | 60 | −20 | 5 months |
| 4** | 0 | 15 | Room temp.‡ | 36 months |
| 7 | 2 | 30 | Room temp.‡ | 36 months |

* The final passage was in a sheep. † Field case (fatal) of anaplasmosis. ** Carrier; source of *A. marginale* used in these studies. ‡ Blood lyophilized.

As has been observed with the electron microscope, no anaplasmata can be seen in untreated cells; the hemoglobin completely masks the parasite. Therefore, some method of hemolysis must be employed. In these studies, hemolysis was generally accom-

* Leitz Ortholux Microscope, and † Heine condenser, manufactured by Ernst Leitz GmbH, Wetzlar, Germany.

** Model PR-1, International Equipment Co., Boston, Mass.

‡ Philips 100 kv. electron microscope, manufactured by Philips, Delft, Holland.

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plished by the use of specific hemolysin, saponin, or by freezing and thawing. In a few instances, water was used. Treatment with specific hemolysin or saponin was preferred, since the rate of hemolysis could be controlled and both the stromata and the parasites remained morphologically unchanged for as long as 48 hours at room temperature. Freezing and thawing produced almost complete hemolysis, but the process could not be observed. The contrast between the Anaplasma and the stroma substance was often poor. In some cases, the preparations improved after standing, while in others the addition of saponin was required. When water was used, hemolysis took place almost immediately. An added disadvantage in using water is that after preparations stand for several hours the anaplasmata become swollen and distorted. The method used most frequently was treatment of the erythrocytes with hemolysin, since it was felt that this was the least drastic method and less apt to alter the anaplasmata.

Unhemolyzed erythrocytes observed with the phase microscope appear as round, bright, highly refractile bodies surrounded by a halo. At the beginning of hemolysis, the erythrocytes appear to shrink momentarily and become dark and opaque. The halos gradually disappear and the cells increase in size. As hemolysis progresses, the cells become lighter and lose their opacity. By the time that hemolysis is complete, the stromata have increased slightly beyond the original size of the erythrocyte and become clear and almost transparent with well-defined membranes. As the hemoglobin leaves the infected cells, the anaplasmata begin to become visible and appear as dark, well-defined bodies when hemolysis is complete (fig. 1, 2).

When observations were made with completely hemolyzed cells from the 10 animals studied (with the single exception of animal 33), few of the anaplasmata were found to be the punctiform bodies one sees in stained preparations. Instead, a variety of structures was encountered, nearly all of the anaplasmata being composed of a knob-like portion, or "head," to which was attached either a ring or a projection like a tail which usually gave the appearance of a match or a straight pin. In fresh blood from animals 31 and 1F, the most commonly observed form was that of a ring, while in the frozen blood from calf 31 and the other animals the majority of the anaplasmata appeared as tail forms. Some tails and rings were delicate, thin, and barely visible, while others were rather thick and approximately the same density as the head. There were also parasites with less compact tails which resembled comets and occasionally dumbbell-like forms, as well as a number

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**Phase contrast photomicrographs of erythrocytes from animals infected with Anaplasma marginale, hemolyzed in the presence of complement with specific hemolysin or with saponin.**

**Fig. 1—Four erythrocytes from animal 31. Cells had been stored in Bovarnick's solution at 5 C. for five weeks and were treated with specific hemolysin. Unhemolyzed erythrocyte (A); hemolysis just beginning (B); hemolysis more advanced, with an Anaplasma barely visible (C); hemolysis almost complete; clearly visible Anaplasma (D). x 1,333.**

**Fig. 2—Same field as figure 1, showing more advanced hemolysis. Erythrocyte still unhemolyzed (A); partial hemolysis (B); almost complete hemolysis (C); complete hemolysis (D); and another cell which moved into the field (E). Anaplasma can be seen in C, D, and E. x 1,333.**

**Fig. 3—Erythrocytes from same preparation as shown in figures 1 and 2. Cells are completely hemolyzed; match and ring forms can be seen. x 1,750.**

**Fig. 4, 5—Erythrocytes from animal 11. Cells had been stored in Bovarnick's solution at −20 C. for 16 months. After thawing they were treated with saponin to produce complete hemolysis. The anaplasmata appear as rings, comets, matches, and bizarre forms, the latter apparently composed of 2 or more parasites. x 1,750.**

**Fig. 6—Erythrocytes from animal 33 which was inoculated with Anaplasma marginale after passage through 5 cattle and 1 sheep. The blood had been stored in Alsever's solution at 5 C. for two days. Washed erythrocytes were then treated with specific hemolysin. The anaplasmata appear as rounded bodies without tails. x 1,750.**

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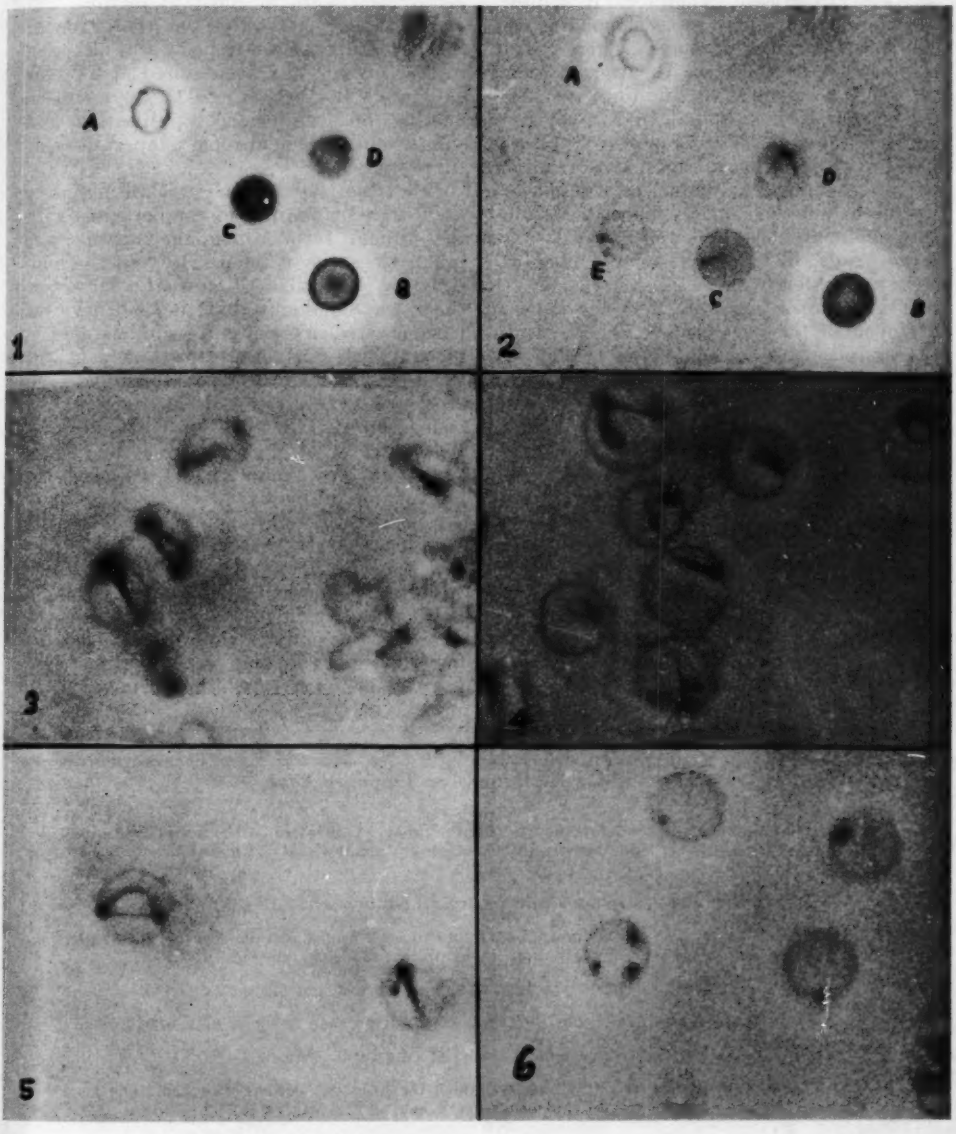
of bizarre structures composed of groups of several heads seemingly joined together by the tails (fig. 3–5). The anaplasmata varied considerably in size, the smallest heads being approximately 0.2 $\mu$ with tails or rings of about 1 $\mu$ in length. The largest parasites had heads 1 $\mu$ in diameter with tails usually 3 to 5 $\mu$ long. Often the tails would extend completely across the cell.

In animal 33, which had an acute infection produced by *A. marginale* after passage in 5 cattle and 1 sheep, there were only a few large ring and tail forms. Most of the parasites appeared as large heads, sometimes doughnut-shaped, without tails or with only rudimentary ones. There were also many small, delicate rings that were circular or, more often, ovoidal in shape.



The latter forms were smaller than those encountered in the blood of the other animals and often appeared to be without heads. However, if observed for sufficient time, a dense, slightly swollen area could usually be seen as the organism turned or changed planes within the stroma. It is well known that *A. marginale* can be maintained in sheep without the animals showing anaplasmata in their erythrocytes or any signs of disease. Therefore, it is interesting that a change in the morphology of the parasite appears to have occurred after one passage in a sheep. An attempt is being made to confirm this finding.

The small rings found in the erythrocytes of animal 33 were observed in the blood of only 1 other animal (1F), in which they were much less abundant. The majority of the anaplasmata in this animal were the commonly observed forms but a few large heads, like those seen in animal 33, were encountered.

Even more remarkable than the variety of forms of the parasite was the fact that the anaplasmata seemed to be motile, exhibiting a swimming movement or amoeboid activity. The head was usually pressed against the cell membrane, often appearing to exert considerable pressure, as though the parasite were attempting to leave the stroma. The ring or tail portion would contract and extend as the organism moved and would appear and disappear as it changed planes within the stroma. It was often observed that a structure which was clearly a ring would turn and appear as a tail or comet, then change again to its original form. Larger, more commonly observed forms varied considerably in their degree of motility both from one sample to another and among individual organisms in a given sample. Motility was particularly marked in freshly drawn blood from animal 31. The large, apparently tail-less forms seen in calf 33 did not seem to be motile, although they could be seen to change position within the stroma. However, the small rings found in the blood of animals 1F and 33 were the most active of all the forms observed and were in constant motion, traversing the whole area of the stromas. Slight motility of the parasite was also seen in blood which had been stored at 5 C. for as long as 40 days, at −20 C. for up to 30 months, or even after lyophilization and subsequent storage at room temperature for three years.

It was not possible to correlate precisely the degree of motility of the parasites with the animal from which the sample was taken, the stage of the disease, nor the temperature and time of storage. However, there was no doubt that the motility was more marked in freshly drawn blood and in blood which had been stored at 5 C. for short periods than in blood which had been frozen or lyophilized and stored for a long time. However, there was also evidence that the addition of certain drugs and vital stains at least temporarily increased the motility of the parasite.

Some extra-erythrocyte forms of varying size were observed in all of the preparations which were studied. They were often seen adhering to the cell membranes as though they were endeavoring to penetrate the stromata. Sometimes they were seen moving about in the fluid in which the cells were suspended. It is difficult to say with

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**Electron photomicrographs of erythrocytes from animal 31 infected with Anaplasma marginale, hemolyzed with specific hemolysin or saponin. Shadow-cast with palladium at arc tangent 2/10.**

**Fig. 7—Saponin hemolysis; Anaplasma is in a marginal position with straight rigid tail. x 30,000**

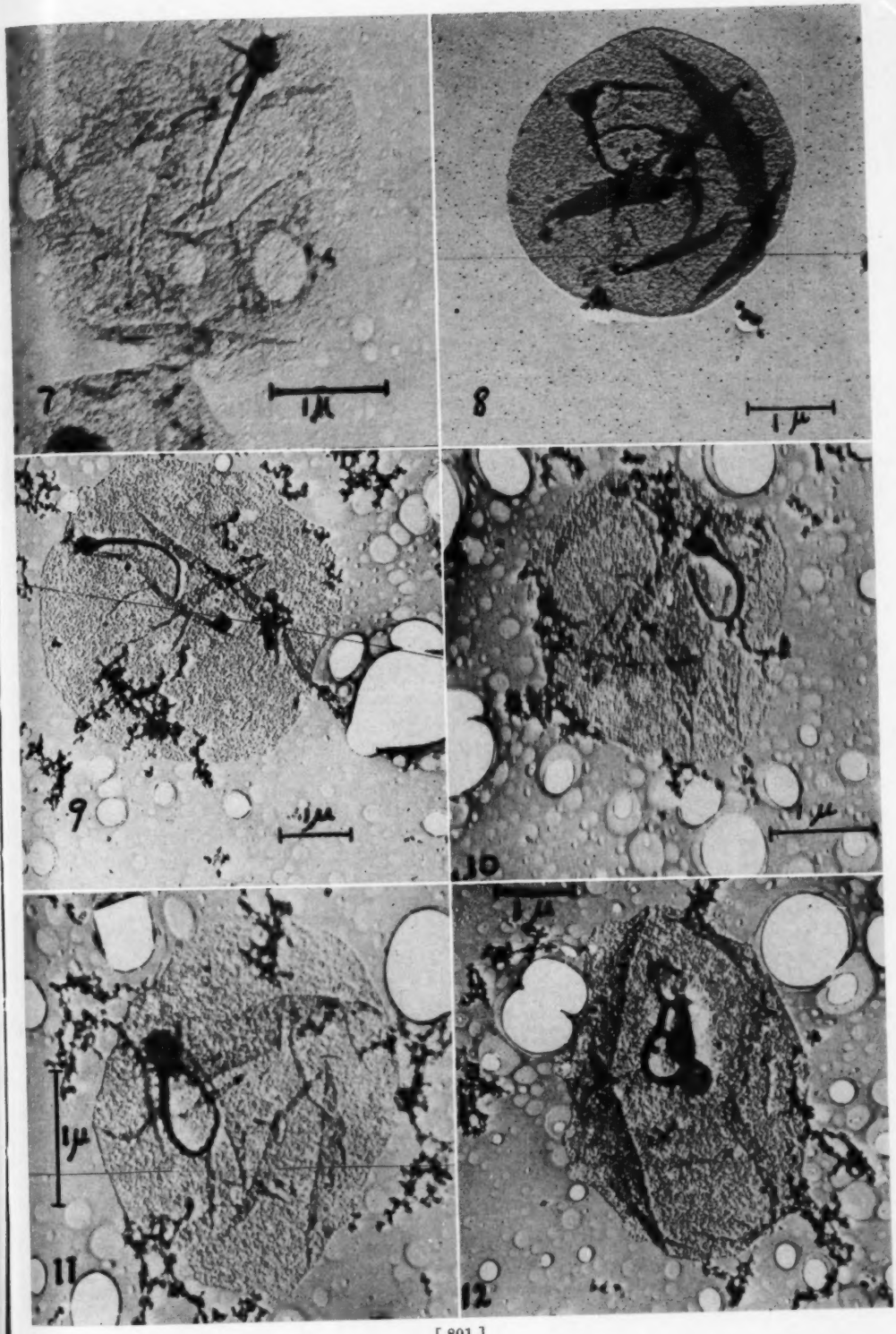
**Fig. 8—Saponin hemolysis; notice internal structure in both head and tail. One of the smaller bodies inside the head appears to be in the process of being released. x 22,500.**

**Fig. 9—Amboceptor hemolysis; the tail is very narrow and curved. x 22,500.**

**Fig. 10—Amboceptor hemolysis; the tail is more curved than in figure 9. x 30,000.**

**Fig. 11—Amboceptor hemolysis; ring form. x 45,000.**

**Fig. 12—Amboceptor hemolysis; ring form in which the head has become detached. The head of another parasite can also be seen behind the ring. x 22,500.**



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certainty that these forms were actually anaplasmata, although they appeared identical to the intracellular bodies. Since they sometimes appeared in large numbers, further studies are being carried out to determine their nature.

Neither anaplasmata nor structures resembling them were found in fresh blood from a healthy carrier, from 3 normal animals, nor in frozen blood from a fourth normal calf. However, in some stromata there were small, round bodies (approximately 0.2 $\mu$) moving about with great rapidity. These were occasionally seen in infected cells as well, but they were easily distinguished from the anaplasmata. These bodies were assumed to be normal constituents of the erythrocytes, perhaps related to the age of the cell, since they were not present in all cells.

*Electron Microscopy.*—In blood from the 10 animals studied, forms identical to those seen in phase microscopy were observed with the electron microscope using erythrocytes hemolyzed either with saponin or with specific hemolysin. The *A. marginale* usually appeared as a marginal body or head of high electron density and a filamentous portion or tail which might be straight and rather thick (fig. 7), slightly curved and slender (fig. 8, 9), or curved to almost form a ring (fig. 10). Ring forms were also observed frequently (fig. 11–15) as well as comet-like structures (fig. 13). In some instances, two parasites seemed to be joined tail to head (fig. 16) or tail to tail, resembling a dumbbell (fig. 17). When the head was observed at high magnification, it was found that the chromatin-like substance was arranged in a dense undivided mass near the center with a number of small rounded bodies around the periphery (fig. 18). This structure of the head has been reported previously by De Robertis and Epstein [6] and Espana.[9] The filamentous portion of the parasite was not of uniform electron density; it sometimes presented an almost beaded appearance (fig. 11, 13, 16).

## DISCUSSION

Although ring and tail forms of *A. marginale* have been reported by several other investigators, this is the first time that these structures have been observed in large numbers and in fresh, unstained erythrocytes. Nor was motility of the parasite observed in earlier work. Further studies will be necessary before the full significance of these findings can be determined. However, they point strongly to the protozoan nature of the parasite.

The observation that the removal of hemoglobin from the erythrocyte is prerequisite to the demonstration of ring and tail forms with phase and electron microscopy may be significant, in that it could explain the usual failure to find these forms in conventionally stained preparations. It is probable that the early workers who reported elongated forms treated their smears in some way which produced hemolysis. This is true of Boynton,[2] who found ring and comet forms only in films which were either fixed with osmic acid and stained with Toisson's fluid or fixed in 1 per cent formalin, then stained with Jenner's and Giemsa's stains.

So far, we have been unable to confirm the findings of Boynton either by following

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**Electron photomicrographs of erythrocytes from animal 31 infected with Anaplasma marginale, hemolyzed with specific hemolysin or saponin. Shadow-cast with palladium at arc tangent 2/10.**

**Fig. 13—Saponin hemolysis; ring and comet-like forms. x 22,500.**

**Fig. 14—Saponin hemolysis; stroma with multiple parasites. x 60,000.**

**Fig. 15—Saponin hemolysis; stroma with multiple parasites at lower magnification than in figure 14. x 20,000.**

**Feg. 16—Saponin hemolysis; stroma containing two parasites coupled head to tail. x 30,000.**

**Fig. 17—Saponin hemolysis; stroma containing two parasites coupled tail to tail. x 30,000.**

**Fig. 18—Anaplasma head at high magnification. The internal structure is in accordance with observations by other workers. x 130,000.**

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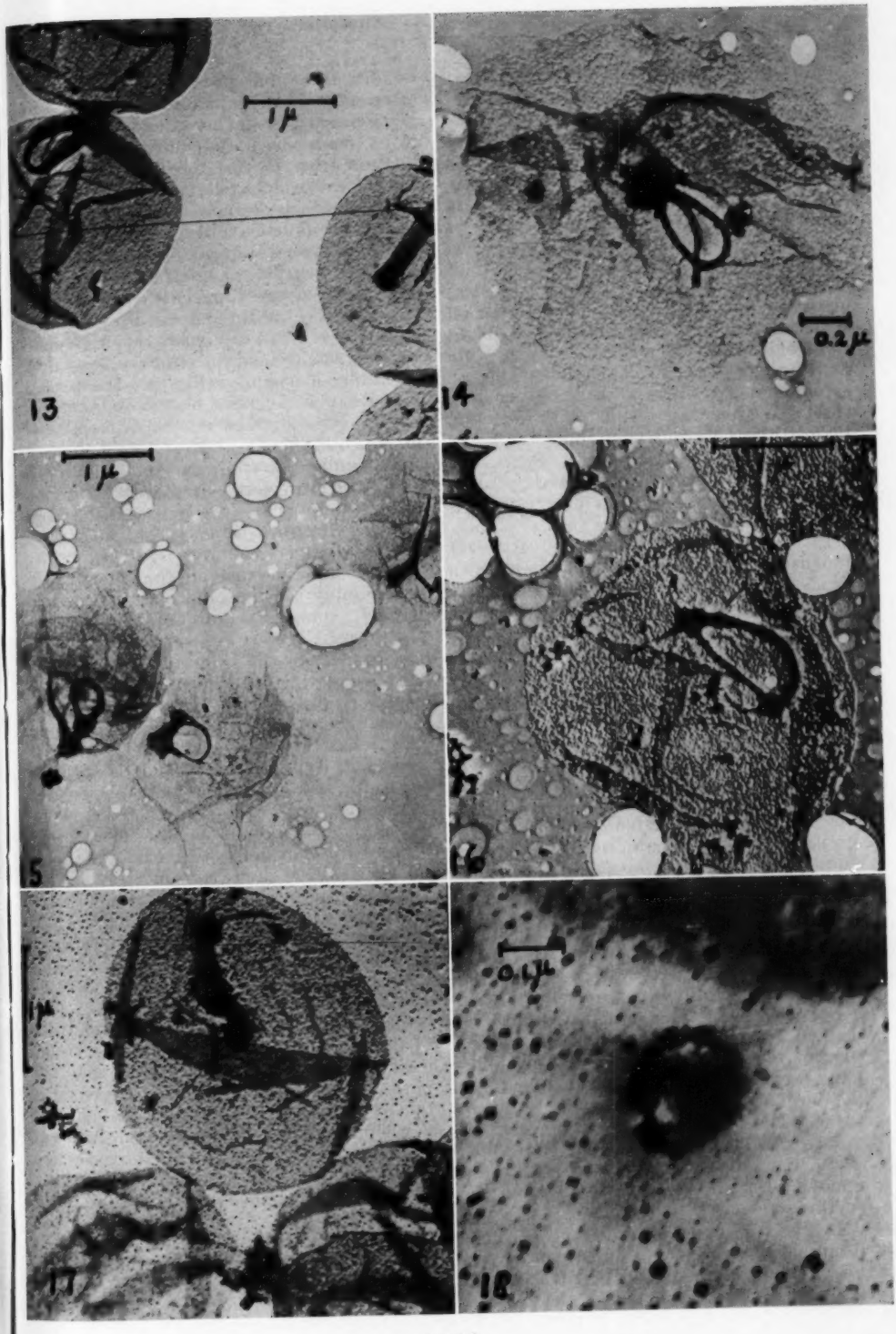
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his technique or by staining previously hemolyzed erythrocytes with Jenner's and Giemsa's stains. Neither did Lotze [16] see these structures in blood which was hemolyzed with 0.5 per cent acetic acid, then stained with Wright's and Giemsa's stains. Using Lotze's technique, we were able to demonstrate with phase microscopy all of the forms previously described in blood hemolyzed by the methods used in this study. Some swelling of the parasites was observed and the heads showed internal structure, but in no instance were the eight "sporoid bodies" described by Lotze seen. However, several small bodies dispersed throughout the stromata were occasionally observed. In smears stained with Wright's followed by Giemsa's stain, only the heads of the anaplasmata were seen. Some, but not all, of the structures described by Lotze were observed. Only a few poorly defined filamentous structures were seen.

In this laboratory, however, Ramirez Valenzuela [18] has demonstrated the various forms seen with phase and electron microscopy using silver impregnation with previously hemolyzed erythrocytes.

It is difficult to explain why De Robertis and Epstein [6] were unable to see the filamentous portion of the parasite with the electron microscope, since the erythrocytes used in these studies were hemolyzed. As for Espana's [9] failure to demonstrate this structure, it was found in more recent studies with phase and electron microscopy that the methods originally used for the preparation of stromata were too drastic. In an effort to remove as much hemoglobin as possible from the cells, the stromata were subjected to repeated washings with distilled water (pH 5.5) and to high-speed centrifugation. This procedure was found to destroy the majority of the rings and tails either partially or completely.[10] When a portion of the filamentous structure remained, it could hardly be recognized as a part of the organism and would certainly have been considered an artifact had one not been aware of the existence of these forms. Thus, it was shown to be essential to produce hemolysis by a method which would not damage the parasite yet would remove most of the hemoglobin from the erythrocyte.

In view of these contradictory findings, it must be assumed that there are other factors involved in the difficulty in demonstrating the filamentous portion of the parasite besides its apparent fragility, its being often situated deep within the cell, and its being masked by the hemoglobin. The chemical composition of the tail must be quite distinct from that of the filamentous portion of the hemoflagellates and the Haemosporidia which stains with Romanowsky's stains. In its low avidity for dyes it would appear to resemble more bacterial flagella. The fact that silver impregnation is the only staining procedure with which structures identical to those seen with phase and electron microscopy have been demonstrated would tend to support this view. However, morphologically the Anaplasma tails bear little resemblance to bacterial flagella but do resemble the organelles of some protozoa.

The finding that *A. marginale* is motile has several interesting implications. In addition to supporting the view that the parasite is a Protozoa, it may help to explain the mechanism of cell invasion. The motility of the Anaplasma may also be important in determining its marginal location within the cell, since as the organism moves, the head is usually seen to push against the cell membrane.

It is tempting to speculate that there may be different strains of *A. marginale*, or different stages in a developmental cycle, which exhibit differences in morphology and motility, and one wonders if *Anaplasma centrale*, which is typically located in a central portion in the infected erythrocyte, may be devoid of the filamentous structure and be nonmotile. Little is known of the virulence of *A. marginale*, but it appears that differences do exist among various isolates of the parasite. However, it is well known that *A. centrale* is of low virulence. Thus, differences in virulence might also be related to the morphology and the motility of the anaplasmata.

The factors which influence the motility of the organism remain to be studied thoroughly. However, it is believed that the period of the disease at which the blood is taken (which would determine the stage of development of the anaplasmata) and possibly the condition of the erythrocytes are important in influencing the motility of the parasite. The methods used in preserving the blood as well as in preparing it for observation are undoubtedly of importance.

Although a variety of forms of the parasite have been observed, the most common and perhaps the only erythrocytic form of

the single parasite may be the ring. The changing of the Anaplasma from one form to another has been observed repeatedly with phase microscopy. In preparations of fresh blood in which a large number of ring forms are seen, anaplasmata resemble young trophozoites of some species of Plasmodia as they appear in stained films. They differ in that the ring portion of the latter stains with Romanowsky's stains, while that of the Anaplasma does not. They also are similar to some members of the family Babesidae, particularly *Gonderia mutans* and *Theileria parva*, again with the same difference in staining properties.

The application of some of the most advanced techniques in microscopy has led us to tentative conclusions akin to those of some of the early workers in the field who, only with a simple optical microscope, a shelf of stains, a keen eye and perseverance, called anaplasmosis "bovine malaria."

## SUMMARY

Examination of hemolyzed erythrocytes from cattle infected with *Anaplasma marginale* by phase contrast and electron microscopy has shown this parasite to be more complex than is generally believed. Ring, match, comet, and dumbbell-like forms have been observed in natural and experimental infections.

*Anaplasma marginale* was found to be motile, a property hitherto undescribed which may be of importance in the classification of the parasite and in explaining the typical marginal position, as well as the mechanism of cell penetration.

A possible explanation is offered to account for the failure of most other workers to describe the forms found in this study.

In the light of these observations it seems justifiable to consider *A. marginale* a true parasite, probably belonging to the Protozoa.

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# The Relationship of Diet to Gastrointestinal Helminth Parasitism in Cattle

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In 1955, Vegors *et al.*[3] reported that yearling cattle on winter pastures in Georgia generally were more adversely affected by parasitism or harbored more worms when slaughtered for market after grazing fescue pastures than after grazing temporary (rye grass, oats, and crimson clover) or crimson clover pastures. They also found, as did Riedel,[2] who used an annual rye grass pasture and a fescue pasture, that after a period of contamination with worm eggs, fescue pastures retained more infective larvae than did the other types of pastures.

In their studies, however, there was little or no opportunity to study the apparent effect of different kinds of pastures on parasitism under conditions where factors such as the quantity of feed consumed and the size and kind of parasite infection could be controlled. Accordingly, the present investigation was undertaken to ascertain the effects of fescue as compared with two other types of forage (alfalfa and a mixture of legumes and grass) on internal parasitism in cattle under conditions where the previously mentioned factors were controlled.

## Materials and Methods

*Diets.*—Four experiments were conducted with cattle, using two diets in each experiment. The daily feed consumption of each animal was controlled and recorded. In experiments 1 to 3, second-cutting fescue hay was compared with U.S. No. 2, 50 per cent green alfalfa hay; in experiment 4, fresh fescue was compared with a fresh legume-grass mixture, predominantly clover, Lespedeza, and orchard grass. The forages were obtained from land which had not been grazed for several years. They were analyzed* by standard agricultural chemical methods, and their composition was recorded (table 1).

*Experimental Animals.*—Twenty Holstein-Friesian steers were used. The control animals of experiments 1, 2, and 3 were used again in experiments 2, 3, and 4, respectively. Except where otherwise stated, the following conditions applied to the cattle. They were born and raised in a barn and were kept there until used. After being weaned, they were fed alfalfa hay. During and between experiments, each animal was penned in a cement-covered concrete stall which was cleaned daily to render extraneous infection negligible.

Six steers (22, 23, 48, 77, 80, and 85) had previously been experimentally infected with *Dictyocaulus viviparus* and 1 (80) had been given a light infection with *Ostertagia ostertagi*. All 6 had eliminated these early infections and showed no residual effects and their growth rates had become normal. Three of these (22, 23, and 80) had been kept in a clean pasture lot for various periods.

Between the end of experiment 3 and the beginning of experiment 4, 2 controls (12 and 48) were transferred to a clean pasture lot for a week. In experiment 4, 2 controls (80 and 85) were transferred from the barn to a clean pasture for 11 days during the preinfection period; for this reason their gain during that period is not shown (table 2).

The animals were on the test diets for 33, 107, 101, and 42 days in experiments 1 to 4, respectively, before any were infected. Then, in experiments 1, 3, and 4, half of the animals on each diet were artificially infected and half were maintained as controls. In experiment 2, there were 2 infected animals and 1 control on each diet.

*Procedure.*—The test animals in each experiment were simultaneously infected from the same batch of larvae, but a different batch was used for each experiment. The dates of infection in experiments 1 to 4 were Oct. 27, 1955, and Jan. 8, April 23, and Aug. 29, 1956, respectively. The infective larvae were washed several times before they were administered *per os* to the animals. The total number of larvae and the number of larvae of *Oesophagostomum radiatum* administered to each animal are mentioned in the titles of tables 3 to 5. The number of larvae of each trichostrongylid species was only approximately determined.

The animals were weighed weekly, and their temperatures were taken weekly or more frequently. At fairly frequent intervals, packed blood cell volume determinations and differential leukocyte counts were made, and the condition of the feces was recorded. The number of helminth eggs per gram (e.p.g.) of feces was determined weekly.

Each infected animal was necropsied at death or at the termination of the experiment, and the worms in the digestive tract were recovered and counted as described by Goldberg and Rubin.[1] Tissue samples from some animals were preserved, sectioned, and examined. In experiment 1, the

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number of lesions caused by the larvae of *Oe. radiatum* was counted in three representative 6-inch portions of the small intestine, in two 12-to-16-square inch portions of the cecum, and in the remainder of the large intestine. From these counts, and from measurements of the total lengths and areas of the parts of the intestines from which the representative samples were taken, the total number of lesions in the intestines was computed. In experiments 2 to 4, the number of these lesions was determined by direct count.

**TABLE 1—Analyses of Feeds Used in Diets (Percentage by Weight)**

| | Experiments 1, 2, 3 | | Experiment 4 | |
|---|---|---|---|---|
| | Fescue hay (%) | Alfalfa hay (%) | Fescue* (%) | Legume-grass* mixture (%) |
| Protein | 15.22 | 17.28 | 9.92 | 15.42 |
| Carbohydrate | 43.05 | 34.29 | 45.97 | 43.67 |
| Fat | 3.38 | 7.15 | 2.64 | 3.22 |
| Ash | 6.67 | 7.16 | 6.91 | 6.02 |
| Crude fiber | 25.62 | 27.70 | 30.24 | 27.74 |
| Water | 6.06 | 6.42 | 4.32 | 3.93 |

* Previously dried for 24 hr. at 110 C.; the fescue lost 56.05 per cent of its weight, and the legume-grass mixture 43.8 per cent.

## CLINICAL OBSERVATIONS

The calves in experiment 1 reacted critically to their infections; 3 died between eight and 12 days after infection, and the remaining 3 became comatose and were killed *in extremis* between 22 and 40 days after infection. In experiments 2 to 4, the animals were much less severely affected; these experiments were terminated by killing the test animals between the seventh and the tenth week after infection.

*Inappetence.*—In general, the infected animals showed about the same degree of inappetence on each diet. In experiment 1, all the infected animals went off feed three to four days after infection and remained off feed until they died or were killed. In experiment 2, fescue-fed calf 16 ate only half of its feed for six days, beginning 24 days after infection, and alfalfa-fed calf 55 ate half its ration 23 days after infection and went off feed almost entirely for the next 23 days; the others did not show inappetence. In experiment 3, fescue-fed animal 5 went completely off feed on the fifth day after infection and consumed only 70 per cent of its feed on the following two days; the animal fed alfalfa never showed inappetence. In experiment 4, none of the animals showed marked inappetence, but five to six days after infection each left 20 to 30 per cent of its fodder and lesser amounts two weeks to a month thereafter.

*Body Temperature.*—All infected animals in experiment 1 developed a high fever. Five days after infection their rectal temperatures ranged from 104.6 to 106.6 F. The fever lasted 16 to 18 days in the animals that survived, and until death intervened in the others. In the other experiments, the temperatures of the cattle remained normal.

**TABLE 2—Feed Consumption (lb.) and Nutritional Efficiency of Experimental Cattle**

| Item | Experiment 2* | | | | Experiment 3† | | Experiment 4** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Fescue hay | | Alfalfa hay | | Fescue hay | Alfalfa hay | Fresh fescue | | Fresh legume-grass mixture | |
| Test animal No. | 2 | 16 | 7 | 55 | 5 | 6 | 22 | 77 | 23 | 84 |
| Feed consumed during preinfection period | 1,497 | 1,417 | 1,492 | 1,485 | 1,428 | 1,426 | 1,540 | 1,120 | 1,540 | 1,120 |
| Gain/100 lb. feed consumed during preinfection period | 10.2 | 11.8 | 15.8 | 13.1 | 13.0 | 12.4 | 3.1 | 2.3 | 2.3 | 4.6 |
| Feed consumed during postinfection period | 791 | 720 | 805 | 446 | 782 | 832 | 1,818 | 1,316 | 1,827 | 1,283 |
| Gain or loss/100 lb. feed consumed during postinfection period | 2.9 | 8.1 | 5.3 | —9.0 | 1.7 | —2.3 | 2.3 | 1.5 | 3.3 | 0.8 |
| Control animal No. | 5 | ..... | 12 | ..... | 48 | 12 | 48 | 85 | 12 | 80 |
| Feed consumed during preinfection period | 1,461 | ..... | 1,505 | ..... | 1,427 | 1,427 | 1,540 | .....‡ | 1,540 | .....‡ |
| Gain/100 lb. feed consumed during preinfection period | 11.1 | ..... | 14.2 | ..... | 16.6 | 13.2 | 5.7 | ..... | 2.7 | ..... |
| Feed consumed during postinfection period | 811 | ..... | 810 | ..... | 834 | 834 | 1,837 | 1,268 | 1,854 | 1,307 |
| Gain/100 lb. feed consumed during postinfection period | 12.2 | ..... | 14.6 | ..... | 10.0 | 12.0 | 2.6 | 4.7 | 5.3 | 5.4 |

The test animals in experiment 1 went off feed soon after infection; the data on gains in weight before infection are given in the text, and the data on changes in weight after infection are given in table 3.

* Preinfection period, 107 days; postinfection period, 57 days. † Preinfection period, 101 days; postinfection period, 55 days. ** Preinfection period, 42 days; postinfection period, 46 days. ‡ Feeding of the test diet was interrupted for 11 days during the preinfection period, when the animal was on clean pasture.

**TABLE 3—Number of Worms Recovered, Lesions Caused by Larvae of Oesophagostomum Radiatum, Worm Eggs per Gram of Feces, and Changes in Weight of Experimental Calves after Administration of 1 Million (200,000 Oesophagostomum Radiatum) Larvae per Head in Experiment 1**

| Item | Alfalfa | Fescue | Fescue | Fescue | Alfalfa | Alfalfa |
|---|---|---|---|---|---|---|
| Calf No. | 17 | 11 | 54 | 13 | 10* | 45 |
| Age at infection (months) | 9 | 9 | 9 | 9 | 9 | 9 |
| Day from infection until death (d) or until killed (k) | 40(k) | 36(k) | 22(k) | 12(d) | 9(d) | 8(d) |
| Worms recovered: | | | | | | |
| *O. ostertagi* | 12,650 | 21,225 | 56,500 | 35,475 | 28,800 | 75,370 |
| *T. axei* | 12,410 | 12,900 | 10,760 | 4,575 | 2,130 | 2,430 |
| *H. contortus* | 0 | 0 | 300 | 300 | 0 | 0 |
| *C. punctata* | 120,720 | 120,375 | 123,610 | 151,850 | ..... | ..... |
| *C. oncophora* | 150 | 20,250 | 17,960 | 34,490 | ..... | ..... |
| Cooperia larvae† | 0 | 2,250 | 4,600 | 22,050 | 1,650 | 169,450 |
| *Oe. radiatum* in lumen: | | | | | | |
| 3rd stage | 0 | 0 | 0 | 150 | 525 | 2,300 |
| 4th stage | 1,200 | 4,800 | 14,250 | 6,395 | 3,000 | 0 |
| 5th stage | 50 | 150 | 0 | 0 | 0 | 0 |
| Total worms recovered | 147,180 | 181,950 | 227,980 | 255,285 | 36,105 | 249,550 |
| Oesophagostomum lesions | 41,500 | 8,000 | 21,000 | 14,000 | Not countable | Numerous, not countable |
| Eggs per gram of feces: | | | | | | |
| Mean | 988 | 2,803 | 10,515 | 0** | 0** | 0** |
| Peak | 1,540 | 5,790 | 10,515 | .... | .... | .... |
| D.I.P.‡ | 33 | 33 | 20 | .... | .... | .... |
| Final | 1,540 | 5,790 | 10,515 | .... | .... | .... |
| Weight changes after infection (lb.) | −98 | −159 | −101 | −25 | 3 | −4 |

* There was loss of worms due to postmortem changes, particularly in the ileum, which fell apart when handled. † Development arrested in early fourth stage in all calves except calf 45, in which the majority were in the late fourth stage and many were ready to molt and enter the fifth stage; *C. punctata* predominant by far, but *C. oncophora* also present; stage of development in calf 10 indeterminable because larvae were disintegrating. ** The minimum prepatent period of the infections is greater than 12 days. ‡ Number of days between infection and peak.

*Condition of Feces.*—The stools of all controls remained normal in all experiments. Observations on the infected animals were as follows:

In experiment 1, diarrhea developed in 5 of the 6 animals in about a week and in the sixth (alfalfa-fed calf 17) in about three weeks after infection. Fresh blood was noticed in the feces of fescue-fed calf 11 two weeks after infection.

In experiment 2, the calves on fescue had diarrhea or passed unduly soft and fetid feces for about a month. Those on alfalfa had diarrhea for two to three weeks.

In experiment 3, the feces of all became unduly soft about a month after infection and remained so for half a month. At this time, the fescue-fed animal developed diarrhea. On the thirty-fifth day, fresh blood and excessive mucus were passed with its feces.

In experiment 4, the stool of legume-grass-fed yearling 84 was intermittently soft for about a month, whereas the feces of the others remained normal.

*Blood Changes.*—Irrespective of diet, the hematocrit values for the cattle were equally affected by comparable parasitism. Of the 3 animals that survived longest in experiment 1, fescue-fed calves 11 and 54 became anemic. During infection, their observed hematocrit values ranged from 15.5 to 24.0 and 20.5 to 31.0, respectively, whereas the hematocrit value for alfalfa-fed calf 17 remained normal. However, this alfalfa-fed calf yielded fewer worms than either of the 2 calves fed fescue (table 3).

In experiment 3, the hematocrit values were decreased equally in the infected animals from 38.0 to 42.0 prior to infection to 29.5 to 30.0 after infection for the fescue-fed animal, and from 37.5—43.0 to 28.0—35.0 for the alfalfa-fed animal. In experiments 2 and 4, the hematocrit values remained normal.

In experiment 1, the 2 fescue-fed calves (54 and 11) showed neutrophilic leukocytosis, 47 and 65 per cent neutrophils, respectively, 19 days after infection, whereas the controls averaged 25 per cent neutrophils. Alfalfa-fed calf 17 had 44 per cent neutrophils 35 days after infection.

In experiment 2, there was a slight increase (up to 9%) of eosinophils in the infected animals on each diet.

In experiment 3, the increase in eosinophils, often associated with helminth infections, was more evident. The fescue-fed infected animal showed an increase from 2 per cent before infection to 29 per cent 52 days afterwards; the maximum value for the control was 8 per cent. In the alfalfa-fed infected animal, the increase was from 1 per cent before infection to 13 per cent 45 days after infection, whereas in the control the count never exceeded 2 per cent.

*Feed Consumption and Weight Changes.*—Equal quantities of fodder were available to the animals on each diet. However, the uninfected animals on fescue consumed 4, 2, and 1 per cent less feed in experiments 1, 2, and 4, respectively, than did those on the other diets. In experiment 3, the uninfected animals on each diet consumed the same amount of feed.

The growth rates of the uninfected animals showed that the alfalfa and legume-grass diets were superior to the fescue diet. On the average, the uninfected alfalfa-fed and legume-grass-fed animals gained about 10 per cent more per pound of feed consumed than did the uninfected fescue-fed animals.

In experiments 1 to 3, the uninfected fescue-fed animals, including the test animals before they were infected, consumed 13,202.0 lb. of hay and gained an average of 12.1 lb. per 100.0 lb. consumed; the uninfected alfalfa-fed animals consumed 13,463.0 lb. and gained an average of 13.6 lb. per 100.0 lb. consumed.

In experiment 4, the uninfected fescue-fed animals, including the test animals before they were infected, consumed 7,305.0 lb. of fodder and gained an average of 3.7 lb. per 100.0 lb. consumed; the uninfected legume-grass-fed animals consumed 7,361.0 lb. and gained an average of 4.0 lb. per 100.0 lb. consumed.

The superiority of alfalfa hay over fescue hay in experiment 1 did not manifest itself in the gains in weight of the calves until they had been on their diets for more than a month. During the 33 days before infection, the calves on fescue gained an average of 15.4 lb. per 100.0 lb. of hay consumed, whereas the calves on alfalfa gained 14.1 lb. per 100.0 lb. consumed. During the entire 75-day period of the experiment, the fescue-fed controls gained an average of 11.3 lb. per 100.0 lb. consumed, whereas the alfalfa-fed controls gained 14.4 lb. per 100.0 lb. consumed.

An analysis was made of the data on nutritional efficiency in experiments 2 to 4 (table 2); the data for calf 55 of experiment 2 were not included because this animal went off feed for a long period. This analysis showed that in each of these experiments the difference between the gain of controls versus the gain or loss of moderately to heavily parasitized animals was greater, usually considerably, than the difference between the gain or loss of infected animals on one diet versus the other. The difference between infected animals on one diet versus the other ranged from 0.02 to 3.95 lb. per 100.0 lb. of feed consumed, while the difference between infected versus control animals, irrespective of diet, ranged from 2.32 to 11.4 lb. per 100 lb. of feed consumed. For infected animals, the difference in gain associated with difference in diet was negligible in experiment 2; in experiment 3, it was in favor of the fescue-fed animal; and in experiment 4, it was in favor of the animals fed the legume-grass mixture.

A similar comparison was not feasible for the animals in experiment 1, because all the infected animals died or were killed *in extremis* within 40 days; their weight changes after infection are shown (table 3). Those fed fescue survived on the average four days longer than those on alfalfa.

*Worm Eggs Passed by Experimental Animals.*—The mean, peak, and final estimates of the number of worm e.p.g. passed by the infected animals are given (tables 3 to 5). In most cases, more eggs per gram were passed in the feces of the animals fed fescue than in the feces of those fed alfalfa, when animals with similar fecal consistencies were compared. In experiment 1, 2 calves fed fescue and 1 fed alfalfa survived long enough for the worms to become gravid; the average number of worm eggs per gram passed by the animals fed fescue was 6.7 times that passed by the animal fed alfalfa. In experiment 2, the average number of worm eggs per gram passed by alfalfa-fed calf 7 was 1.4 times that passed by fescue-fed calves 2 and 16. The data for alfalfa-fed calf 55 were not included in this calculation because the animal was off feed for 23.5 days, and a peak of 29,400 eggs per gram was reached because of the small quantity of feces passed. Toward the end of the experiment when it

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went back on feed, the number of worm eggs per gram was comparable to that of the other infected animal fed alfalfa. Apparently, the worms were not seriously harmed by the failure of the host to consume its allotted ration. In both experiments 3 and 4, the average number of worm eggs per gram of feces passed by the animals fed fescue was 1.9 times that of the animals fed alfalfa.

### NECROPSY FINDINGS

*Gross Pathology.*—In experiment 1, the 3 animals that died and the 1 killed 22 days after infection (table 3) showed the severest pathological changes, and the severity of the changes apparently was not correlated with diet.

The 3 animals that died showed severe hemorrhagic enteritis of the ileum and large intestine, presumably caused by invasion of the intestinal mucosa by larvae of *Oe. radiatum* and consequent bacterial infection. The mesenteries of these animals were edematous, icteric, and hemorrhagic; the mesenteric lymph nodes were enlarged and edematous; the abomasums were edematous and inflamed and showed numerous petechiae; and the livers showed fatty degenerative changes. The first calf that died showed severe peritonitis and bloody ascites. Diffuse hemorrhage was present under the serosa of the ileum and large intestine of this calf, and petechiae and ecchymoses were present under the serosa of the other 2 calves. The kidneys of 1 were markedly hyperemic.

In the 2 animals killed 36 to 40 days after infection, many of the intestinal lesions had become nodular and filled with pus, and the portions of the intestines involved had become thickened. The mesenteries were edematous and adhered in some areas to the viscera. The lymph nodes and Peyer's patches contained pus. The abomasum of 1 calf showed numerous minute pustular lesions. A severe and extensive nephritis, presumably bacterial in origin, had developed in the animal killed 22 days after infection. The 2 animals that survived longest were emaciated.

The pathological changes in the infected animals of experiments 2 to 4 were much less severe. All animals showed slight gastritis, particularly in the pyloric region. In the abomasums there were a few ulcerated lesions, 2 mm. in diameter. The small intestines and ceca were slightly inflamed, particularly around lesions caused by nodular worms. The walls of the small intestines were thickened in some sections. In experiment 4, the younger animal fed fescue had less fat than the younger ani-

**TABLE 4—Number of Worms Recovered, Lesions Caused by Larvae of Oesophagostomum Radiatum, and Worm Eggs per Gram of Feces Passed by Experimental Animals after Administration of 400,000 (4,600 Oesophagostomum Radiatum) and 500,000 (75,000 Oesophagostomum Radiatum) Larvae per Head in Experiments 2 and 3, Respectively**

| Item | Experiment 2 | | | | Experiment 3 | |
|---|---|---|---|---|---|---|
| | Fescue | Alfalfa | Alfalfa | Fescue | Fescue | Alfalfa |
| Animal No. | 16 | 55 | 7 | 2 | 5 | 6 |
| Age at infection (months) | 11.3 | 11.3 | 11.8 | 12.0 | 15.0 | 15.0 |
| Days from infection to necropsy | 73 | 71 | 63 | 59 | 58 | 56 |
| Worms recovered: | | | | | | |
| *O. ostertagi* | 9,000 | 3,600 | 6,450 | 16,600 | 4,025 | 4,650 |
| *T. axei* | 3,100 | 24,150 | 17,325 | 14,500 | 76,390 | 41,875 |
| *H. contortus* | 0 | 0 | 0 | 0 | 3,980 | 906 |
| *C. punctata* | 9,000 | 19,350 | 13,500 | 7,500 | 6,300 | 7,400 |
| *C. oncophora* | 1,350 | 225 | 675 | 0 | 0 | 12 |
| *Oe. radiatum* in lumen: | | | | | | |
| 4th stage | 0 | 6 | 0 | 0 | 24 | 0 |
| 5th stage | 72 | 108 | 51 | 138 | 24 | 48 |
| *Oe. radiatum* larvae in wall: | | | | | | |
| 3rd stage | 105 | 70 | 50 | 50 | 1,020 | 130 |
| 4th stage | 245 | 180 | 455 | 155 | 680 | 750 |
| Total worms recovered | 22,872 | 47,689 | 38,506 | 38,943 | 92,443 | 55,771 |
| Oesophagostomum lesions | 775 | 815 | 1,100 | 1,100 | 2,912 | 1,427 |
| Eggs per gram of feces: | | | | | | |
| Mean | 166.3 | 5,180* | 226 | 165.5 | 329 | 177 |
| Peak | 300 | 29,400* | 350 | 447 | 710 | 254 |
| D.I.P.† | 43 | 43 | 36 | 16 | 22 | 22 |
| Final | 83 | 106 | 100 | 89 | 264 | 241 |

* Animal off feed. † Number of days beween infection and peak.

mal fed the legume-grass mixture. Both older animals had an abundance of fat.

*Lesions and Worms in Wall of Digestive Tract.*—In experiment 1, many *O. ostertagi* were embedded in the wall of the abomasums of the calves necropsied up to 22 days after infection. Numerous minute, open, pustular lesions also were present 22 days after infection. However, the data obtained from these few animals were not sufficient to indicate whether the number of embedded Ostertagia was correlated with diet. Few such worms were present in the abomasums of the calves necropsied later in this experiment, and none were seen in the animals in experiments 2 to 4.

The numbers of intestinal lesions caused by *Oe. radiatum* were not markedly different in the animals on the different diets. In experiment 1, numerous bloody lesions, caused by larvae of *Oe. radiatum*, were observed in the posterior half of the small intestines and in the ceca of the calves that died up to 12 days after infection. Some hemorrhagic lesions were present in the intestines of those killed 22 to 40 days after infection, but most of the lesions were discolored, indicating old hemorrhage, and many were filled with pus and contained larvae of the nodular worm. In experiments 2 to 4, most of the lesions were of the last two types.

The nodular lesions which contained pus and were 3 to 6 mm. in diameter were raised 2 to 5 mm. above the surface of the mucosa, whereas the bloody lesions, which were smaller, were raised only 1 mm. and the discolored, old hemorrhagic lesions were flush with the surface. Small, low, clear nodules indicated healed lesions, and hence were not counted (tables 3–5).

The number of nodular worm larvae in the intestinal wall of the infected animals in experiments 2 to 4 was estimated from the number of larvae recovered by dissection of 20 nodular worm lesions taken from each animal (tables 4, 5).

*Worms Recovered from Lumen of Digestive Tract.*—The interval between infection and necropsy and the number of worms recovered from each of the infected animals in experiments 1 to 4 are recorded (tables 3–5). Except where otherwise specified, the following comparisons are based on the number of worms recovered from calves 11 and 17, which survived longest and for about the same length of time, in experiment 1, and on those recovered from all of the animals in experiments 2 to 4, inclusive.

Sixteen per cent more worms were recovered from the animals fed fescue than from those fed alfalfa or the legume-grass mixture. Usually, more of each species were recovered from the animals fed fescue than from those fed the other diets.

In experiments 1, 2, and 4, the animals fed fescue yielded 1.68, 2.54, and 1.06 times as many *O. ostertagi*, respectively, as did the animals on the other diets, but in experiment 3 the animal fed alfalfa yielded 1.15 times as many as did the animal fed fescue.

**TABLE 5—Number of Worms Recovered, Lesions Caused by Larvae of Oesophagostomum Radiatum, and Worm Eggs per Gram of Feces Passed by Experimental Animals after Administration of 700,000 (7,000 Oesophagostomum Radiatum) Larvae per Head in Experiment 4**

| Item | Legume-grass | Fescue | Fescue | Legume-grass |
|---|---|---|---|---|
| Animal No. | 23 | 22 | 77 | 84 |
| Age at infection (months) | 26 | 26 | 12 | 11 |
| Days from infection to necropsy | 56 | 54 | 50 | 48 |
| Worms recovered: | | | | |
| *O. ostertagi* | 9,025 | 10,125 | 12,125 | 11,775 |
| *T. axei* | 100 | 400 | 1,350 | 825 |
| *H. contortus* | 0 | 0 | 0 | 4 |
| *C. punctata* | 17,850 | 15,525 | 6,400 | 3,600* |
| *C. oncophora* | 48 | 1,350 | 0 | 0 |
| *Oe. radiatum* in lumen† | 96 | 804 | 1,392 | 456 |
| *Oe. radiatum* larvae in wall | | | | |
| 3rd stage | 335 | 470 | 170 | 140 |
| 4th stage | 285 | 235 | 110 | 0 |
| Total worms recovered | 27,739 | 28,909 | 21,547 | 16,800 |
| Oesophagostomum lesions | 1,080 | 1,640 | 1,185 | 740 |
| Eggs per gram of feces: | | | | |
| Mean | 99 | 108 | 157 | 38 |
| Peak | 142 | 225 | 291 | 67 |
| D.I.P.** | 29 | 54 | 50 | 48 |
| Final | 122 | 225 | 291 | 67 |

* Including 150 Cooperia larvae showing arrested development in fourth stage. † All fifth stage. ** Number of days between infection and peak.

In experiments 1, 3, and 4, the animals fed fescue yielded 1.04, 1.82, and 1.88 times as many *Trichostrongylus axei*, respectively, as did those on the other diets, but in experiment 2 the animals fed alfalfa yielded 2.35 times as many as did the animals fed fescue.

In experiment 1, 300 *Haemonchus contortus** were recovered from each of 2 ani-

* Throughout this paper, *Haemonchus contortus* is used in *sensu lato*.

mals fed fescue, whereas the animals fed alfalfa yielded none; in experiment 3, the animal fed fescue yielded 4.39 times as many *H. contortus* as did the animal fed alfalfa.

In experiments 1, 2, and 4, the fescue-fed animals yielded 135.0, 1.5, and 28.1 times as many *Cooperia oncophora*, respectively, as did the animals on the other diets.

In experiments 1, 3, and 4, the fescue-fed animals yielded 3.96, 1.88, and 2.42 times as many *Oe. radiatum*, respectively, as did the animals on the other diets; in experiment 2, the alfalfa-fed animals yielded 1.20 times as many as did the fescue-fed animals.

Some data, not shown in the tables, were obtained on the loss of worms occurring at the time of necropsy. In the four experiments, there were 24 instances of worms recovered on their way out of the host. In 42 per cent of the instances, the number of such worms was less than 1 per cent of the number in the normal location; in 75 per cent it was less than 5 per cent; and in 8 per cent it was 10 per cent or more. The number passing out did not appear to be correlated with diet.

*Development of Parasites.*—Transition of the larvae of *Oe. radiatum* from the third to the fourth stage occurred on the ninth day after infection and that from the fourth to the fifth stage usually occurred after 36 days. Fourth-stage larvae were last recovered from the lumen of the small and large intestines 40 and 71 days, respectively, after infection. Although there was some difference in the rate of development of *Oe. radiatum* in different animals, it was not correlated with diet.

Since retardation of the development of worms has been interpreted as evidence of resistance of the host to infection, it is of interest that some larvae of Cooperia spp. showing arrested development in the early fourth stage were recovered up to 48 days after infection. The number of larvae of Cooperia spp. recovered from each animal in experiment 1 is given (table 3). In experiment 4, approximately 150 larvae of Cooperia spp. showed arrested development in the small intestine of legume-grass-fed yearling 84. The number recovered appeared to be correlated with magnitude of the infection and not with diet, and it decreased with time after infection. No Cooperia spp. showing arrested development were recovered in experiments 2 and 3.

*Age of Host in Relation to Parasitism and Diet.*—In experiments 1, 2, and 3, the animals were approximately the same age (tables 3 and 4), but in experiment 4, 1 of the test animals was 11 months old, another 12 months old, and 2 were 26 months old at the time of infection. The difference in age, as well as the difference in diet, appeared to be a factor in the difference in weight gain and number of parasites recovered from the animals in experiment 4.

In the postinfection period, the average gain of the younger infected animals was one third as much per pound of feed consumed as in the preinfection period, whereas the average gain of the older ones per pound of feed was approximately equal in these two periods. The average number of worm eggs per gram of feces passed by the older animals was 1.06 times that passed by the younger ones.

The total number of worms recovered from the older animals was greater than that recovered from the younger ones, but the younger animals yielded more worms of each species except *C. punctata* and *C. oncophora*. The younger animals yielded 1.2, 4.35, and 2.1 times as many *O. ostertagi*, *T. axei*, and *Oe. radiatum*, respectively, as did the older animals. The older animals yielded 3.4 times as many *C. punctata* as did the younger animals. One of the older animals yielded 48 and the other 1,350 *C. oncophora*, whereas none were recovered from the younger animals. The older animals had 1.4 times as many nodular worm lesions and three times as many larvae in the wall of the intestine as did the younger animals.

## DISCUSSION

The analyses of the diets indicated that the alfalfa hay contained more protein and fat and less carbohydrate than the fescue hay and that the legume-grass mixture contained much more protein and slightly more fat than the fescue grass. The weight gained by the uninfected animals showed that the alfalfa and legume-grass diets were superior to the fescue diet. However, in experiment 1, the superiority of alfalfa hay to fescue hay was not manifested in greater rate of gain until the animals had been on the diets for more than a month.

As previously stated, in work which involved cattle on pasture, Vegors *et al.*[3]

found that those on fescue generally were more adversely affected by parasitism than those on better diets. In the present study, where the same amount of feed was made available to each animal, in comparable moderate-to-heavy infections, alfalfa and a legume-grass mixture were not sufficiently superior to fescue to prevent adverse effects caused by the parasites. The clinical signs were equally severe in comparable infections. In general, comparably infected animals showed about the same degree of inappetence, and the stools became abnormal about the same time and to the same degree, and remained so for about the same period on each diet. The effects on weight, hematocrit values, and severity of the pathological changes were about the same in comparably infected animals, irrespective of diet. There was no marked difference between the fescue-fed animals and those on the other diets in the numbers of *Oe. radiatum* lesions and larvae occurring in the wall of the intestine.

The development of a high fever by the infected animals in experiment 1, the death of 3 animals within 12 days, and the comatose condition of the others within 40 days after infection were probably due to a bacteremia apparently resulting from bacterial invasion of the numerous lesions produced in the intestines by the larvae *Oe. radiatum*. Many more of these larvae were administered to the animals in experiment 1 than to those in the other experiments, and many more lesions due to this parasite were present in the animals of experiment 1. In the other experiments, there were no marked complications and the temperature of the animals remained normal.

On the average, the number of worms recovered from the animals fed fescue was greater than that recovered from those on the better diets. This is in agreement with the findings of Vegors *et al.*[3] In the majority of instances, the animals fed fescue also yielded a greater number of worms of each species than did the animals on the other diets.

In the majority of cases, more worm eggs were passed by the animals fed fescue than by those on the other diets. Consequently, if the animals had been on pasture, those on fescue would have contributed more toward contaminating the pasture than those on the other diets. This finding is of particular interest in the light of the previously mentioned reports of Vegors *et al.*[3] and Riedel[2] that more infective larvae were found on fescue pastures than on comparably grazed pastures of other types.

## SUMMARY AND CONCLUSIONS

The relationship between diet and gastrointestinal helminth parasitism in cattle was studied in four experiments. The parasites used were *Oesophagostomum radiatum, Cooperia punctata, Cooperia oncophora, Ostertagia ostertagi, Haemonchus contortus,* and *Trichostrongylus axei.* The diets compared were (1) second-cutting fescue hay versus U.S. No. 2, 50 per cent green alfalfa hay and (2) fresh fescue, slightly mixed with other herbs, versus a fresh legume-grass mixture. Analyses of the feeds showed that the alfalfa hay contained more protein and fat and less carbohydrate than the fescue hay and that the legume-grass mixture contained much more protein and slightly more fat than the fresh fescue.

Comparable parasite-free cattle were placed on each diet. Sixteen animals were infected and 14 were maintained as controls. In each experiment, equal aliquots of infective larvae from the same batch were administered simultaneously to the test animals on each diet. Extraneous infection was virtually prevented. The infections produced were moderate to heavy.

Various comparisons were made between the animals on each diet during preinfection and postinfection periods. The uninfected alfalfa-fed and legume-grass-fed animals gained 1.1 times as much per pound of feed consumed as did the uninfected fescue-fed animals. However, in experiment 1, more than a month was required to demonstrate the nutritional superiority of alfalfa hay to fescue hay. In general, infected animals showed about the same degree of inappetence on each diet.

In comparably infected animals, there did not appear to be a significant difference in the effect of the fescue and other diets on weight gains. The effect of moderate-to-heavy parasitism on rate of gain was usually considerably greater than the effect of the difference in diets.

In comparable infections, clinical signs were similar in severity, irrespective of diet. The feces of infected animals on the different diets became abnormal at about the same time and to the same degree, and

XUM

remained so for about the same period. The hematocrit values were correlated with the degree of parasitism rather than with the diet.

On the average, approximately 1.8 times as many eggs per gram were passed in the feces of the animals fed fescue as in the feces of the animals on either of the other two diets.

If the comparison is limited to the numbers of parasites recovered from those animals which were killed at comparable intervals after infection, 16 per cent more worms were recovered from those fed fescue than from those on the other diets; the animals fed fescue also generally yielded more worms of each species than did those on the other diets.

The rate of development of the worms did not appear to be significantly affected by diet.

The number of worms on the way out of the host at the time of necropsy did not appear to be correlated with diet.

There was no marked difference in the number of intestinal lesions caused by the nodular worm, *Oe. radiatum*, or in the number of larvae in the intestinal wall in the animals on the different diets.

In the one experiment in which cattle in two different age groups were used, differences in the degree and severity of parasitism apparently were correlated with age, as well as with diet.

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# Multiplication and Cytopathogenic Effect of Foot-and-Mouth Disease Virus in Cultures of Fetal Rabbit Lung Cells

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FOOT-AND-MOUTH disease virus (FMDV) has been propagated successfully in cultures of various animal tissues.[1,3-5] These tissues were derived from naturally susceptible hosts, *i.e.*, cattle, sheep, and pigs. In this introductory paper the specific cytopathogenic effect of FMDV, type C, in cells of the fetal rabbit lung is described. The rabbit is naturally insusceptible to this agent, but can be infected experimentally intradermally or by scarification, especially with guinea pig-adapted virus.

## MATERIALS AND METHODS

The lung tissue was removed aseptically from rabbit fetuses of 20 to 25 days' gestation. The tissue was minced with scissors and then was washed twice with neutralized Hanks' balanced salt solution (BSS) containing antibiotics (penicillin, 50 units; streptomycin, 50 $\mu$g.). The tissue was then treated with 0.25 per cent trypsin in isotonic phosphate buffer solution according to the following procedure: To the fragments, an aliquot of the solution was added, which was removed after five minutes and discarded. Tissue fragments were then exposed successively at room temperature to six aliquots of the solution, each of which was allowed to react for 15 minutes while the preparation was slowly agitated with a magnetic stirrer. The fluids were combined as removed and kept at 4 C. until the last was added.

From the pool of fluids, the cells were sedimented by centrifugation at 500 to 600 r.p.m. for 30 minutes and resuspended in growth medium to yield a concentration of 300,000 to 350,000 cells per milliliter. This suspension was stirred for 20 minutes with a magnetic stirrer before it was distributed in volumes of 1 ml. to a series of culture tubes. Tubes were slanted and maintained in a stationary position at 37 C.

The growth medium consisted of 79.48 per cent neutralized BSS; 20 per cent inactivated horse serum; 0.5 per cent lactalbumin hydrolysate; 0.02 per cent yeast extract; and antibiotics as in the previously mentioned BSS. The medium was changed at intervals of one week. The cultures were ready for use eight to ten days after incubation at 37 C.

Fluid from cultures of bovine kidney cells infected with strain E 35-A of FMDV, type C, was used as the inoculum to initiate a series of passages of the virus in fetal rabbit lung cells. Antiserum against FMDV was prepared in guinea pigs and used to demonstrate the specific nature of the cytopathic changes observed after inoculation of the virus.

Cultured cells were fixed, embedded, and stained according to the procedure described by Enders and Peebles.[2]

## EXPERIMENTAL PROCEDURE AND RESULTS

Following inoculation of the virus into cultures of trypsinized fetal rabbit lung cells, cytopathic changes were observed after 24 to 30 hours. These consisted predominantly of rounding of the cytoplasm and nuclear pyknosis. Certain of the affected cells were distinguished by elongated "beaded" cytoplasmic processes. About the periphery of others, numerous small spherical cytoplasmic masses presented a conspicuous feature. Eventually, disintegration of all the cells ensued. Intranuclear or intracytoplasmic inclusions were not observed.

These changes (fig. 1, 2) appeared in each of 12 serial passages of the agent in rabbit cell cultures. As inoculum in each passage, with the exception of the first, 0.1 ml. of the fluid was used, diluted $10^{-3}$ from cultures of the preceding passage. The infectivity titer of fluid from the twelfth passage was determined as $10^7$ t.c.d.$_{50}$ per milliliter.

That the cytopathic changes were the result of infection by the virus was demonstrated by the capacity of specific guinea pig antiserum to prevent their development when mixed with infected tissue culture fluid (fig. 3, 4). These photographs are of fetal rabbit cell cultures that were exposed two days previously to a mixture of diluted anti-FMDV, guinea pig serum, and fluid of the fifth virus passage. These cells in all respects resembled those in uninoculated cultures maintained under similar conditions.

Experiments are in progress to determine whether any modification in virulence of the virus may have occurred during the series of passages in the cells of this unnatural host.

From the Instituto Bacteriológico de Chile, Santiago, Chile.

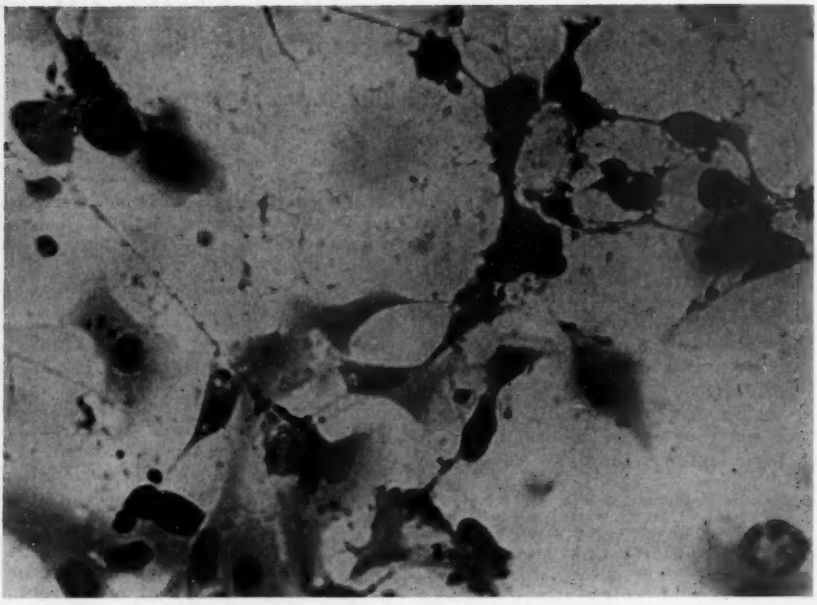

Fig. 1—Appearance of fetal rabbit lung cells in an eight-day culture inoculated 48 hours earlier with foot-and-mouth disease virus. Inoculum: 0.1 ml. fluid diluted $10^{-5}$ from the fifth passage of the virus in rabbit lung cell cultures mixed with an equal volume of normal guinea pig serum diluted 1:8. H & E stain; x 200.

Fig. 2—A virus-affected cell from the same preparation as shown in figure 1. Notice spherical cytoplasmic processes and nuclear changes. H & E stain; x 400.

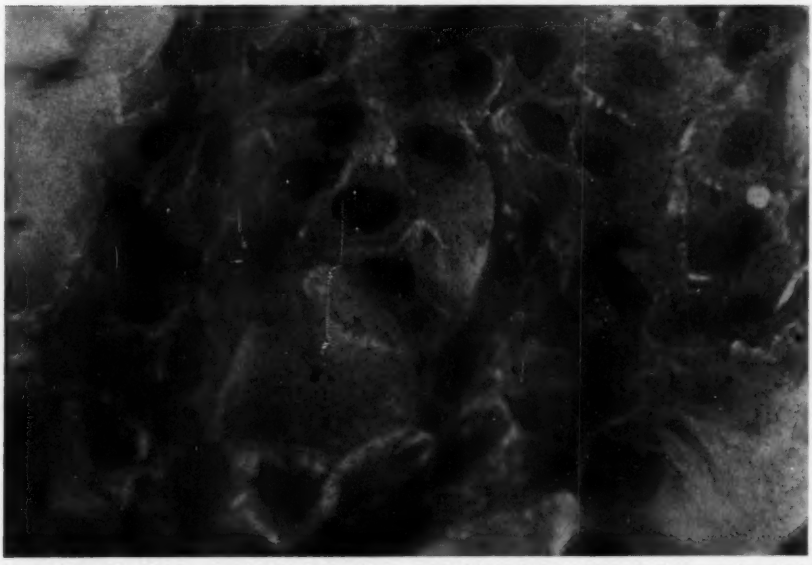

Fig. 3—Normal appearance of cells in a culture comparable to that shown in figure 1, inoculated 48 hours earlier with the same quantity of foot-and-mouth disease virus and mixed with an equal volume of anti-foot-and-mouth disease virus, type C, guinea pig serum diluted 1: 8. H & E stain; x 200.

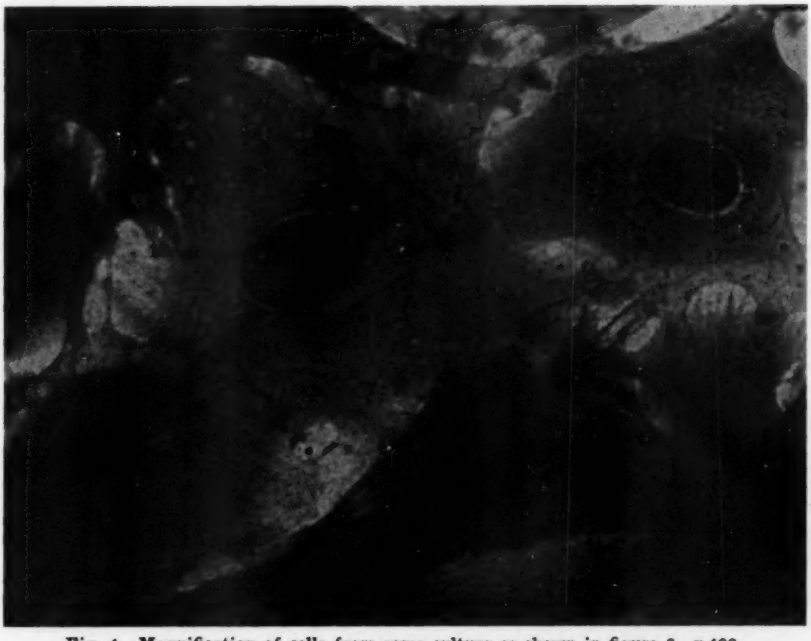

Fig. 4—Magnification of cells from same culture as shown in figure 3. x 400.



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SUMMARY

The virus of foot-and-mouth disease multiplies readily and induces degenerative changes in cultures of fetal rabbit lung cells.

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# Experimental Viral Bovine Mastitis

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ALTHOUGH MICROBIAL INFECTIONS are considered to be a common cause of inflammation of the mammary gland, no single etiological agent has been recognized as common to all cases of bovine mastitis. Mastitis may develop in the absence of pathogenic microorganisms at the onset of lactation and as a result of an injury. Broadhurst [4] suggests that the general acceptance of bacteria as the significant pathogen in mastitis and the use of bacterial methods in diagnosis may have inhibited the search for other agents.

This paper describes the production of mastitis by the introduction of certain viruses into the bovine mammary gland. Development of methods of detecting the presence of these agents in the udder secretions was an essential part of the study.

## REVIEW OF LITERATURE

The first evidence cited by Munch-Petersen [16] that mastitis was of microbial origin was that of Nocard and Mollereau in 1885, Bang in 1889, and Kitt in 1891. The microorganisms most commonly associated with mastitis vary from state to state. In New York and Connecticut, *Streptococcus agalactiae* is associated with the majority of mastitis cases. In Iowa, Illinois, Wisconsin, Ohio, Indiana, and the west coast area, staphylococci are associated with the majority of cases of mastitis.[24]

Several authors [1, 2, 7–10, 17, 19] have described mastitis in which no microorganisms could be associated with the inflammatory process. Some of these authors [8, 10, 17, 19] suggested that a virus could be the etiological agent. Petersen *et al.*[17] stated that the microscopic lesions in the idiopathic cases which he studied were consistent with a virus infection. Attempts to isolate viruses from cases of mastitis have been described in two papers. Broadhurst *et al.*[4] reported the presence of inclusion bodies in the cells contained in milk, in tissue culture cells, and in tissues from mice injected with "infected" milk. Bushnell [5] sought to demonstrate a virus in cases of typical clinical mastitis by injecting milk from the animals onto the chorioallantoic membrane of embryonating eggs. No evidence of viral multiplication was found in the initial cultures or in subcultures.

Mastitis has been associated with some virus diseases, but it has not been demonstrated that these viruses were primary invaders of the mammary gland. Strozzi and Ramos-Saco,[23] Brandly *et al.*,[3] and Heiny [6] have all reported teat lesions and associated mastitis in cases of vesicular stomatitis. One of us (B.C.E.) has seen bovine mastitis associated with foot-and-mouth disease in Kenya. Personal communications from several Wisconsin practicing veterinarians indicate that mastitis is often associated with cases of so-called cowpox.

Most investigators are of the opinion that the mastitis associated with these diseases is due to the lesions produced on the end of the teat, facilitating the entrance of bacteria into the udder. Mitchell *et al.*[11–15] have demonstrated that such viruses as influenza (PR-8) and Newcastle disease will grow and multiply when introduced into bovine and caprine mammary glands. These experiments were designed to determine whether such viruses could be altered or propagated by passage in these glands. No mention was made of any inflammatory processes.

## MATERIALS AND METHODS

*Experimental Animals.*—Nineteen Holstein-Friesian cows, 3 to 5 years old, were used in this study. Two previous herd tests indicated an absence of *Str. agalactiae* or coagulase-positive staphylococci from all cows in the herd. Those lactating had been lactating for approximately one year.

The mammary glands of 8 cows (5 lactating and 3 nonlactating) were inoculated with vesicular stomatitis virus via the streak canal. Two diagonally situated quarters were inoculated on each animal. The remaining two quarters served as uninoculated controls. In the same manner, 6 cows (4 lactating and 2 nonlactating) were inoculated with vaccinia virus and 3 cows (2 lactating and 1 nonlactating) with Newcastle disease virus. Two cows used as inoculated controls were injected with allantoic fluid from 12-day embryonating chicken eggs or with a 10 per cent suspension of chorioallantoic membranes in nutrient broth introduced via the streak canal. Bacteriological tests, leukocyte counts, and modified Whiteside tests were performed on milk from all quarters several times prior to inoculation of the cows.

From the Department of Veterinary Science, University of Wisconsin, Madison. Published with the approval of the director of the Wisconsin Agricultural Experiment Station as paper NS 263. Dr. Easterday is now at the Virus and Rickettsia Division, Fort Detrick, Md., and Dr. Simon is at the Cancer Research Laboratory, University of Florida, Gainesville.

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Rabbits weighing 4 to 5 kg. were used to confirm the presence of vaccinia virus. Intradermal inoculations were made on either side of the dorsal midline with either milk or egg fluids.

White CFW mice weighing 14 to 16 Gm. were obtained from the laboratory mouse colony and were inoculated intracerebrally as an assay for vesicular stomatitis virus.

Fertile White Leghorn chicken eggs were obtained from two local hatcheries and incubated in the laboratory for the required number of days. The embryonating eggs were used for virus production, virus assay, and virus neutralization tests.

*Viruses Used.*—Vaccinia Virus.—The International Health Department (IHD) strain of vaccinia virus (VAC) was used in these experiments. This virus grows when inoculated onto the chorioallantoic membrane of 12-day embryonating chicken eggs, into the yolk sac of 8-day embryos, into the allantoic chamber of 9-day embryos, intracerebrally into 14- to 16-Gm. mice, or intradermally in rabbits.

Vesicular Stomatitis Virus.—The New Jersey (NJ) type of vesicular stomatitis virus (VSV) was used in these experiments. This strain was isolated from an animal with vesicular stomatitis in Rusk County, Wisconsin, in 1949, by Brandly and Hanson.[3] Vesicular stomatitis virus grows in the allantoic chamber of 9-day embryonating eggs to a titer of approximately $1 \times 10^8$ embryo $l.d._{50}$ per milliliter of allantoic fluid. All specific deaths occur within 96 hours postinoculation, with the majority of deaths occurring within 50 hours.

Newcastle Disease Virus.—The Roakin strain* of Newcastle disease virus (NDV) was used. It produces a mild systemic reaction with low mortality (1–2%) in month-old chickens. The virus usually has a titer of $1 \times 10^9$ embryo $l.d._{50}$ per milliliter of allantoic fluid when introduced into the allantoic chamber of 10-day embryonating eggs.

*Collection of Milk Samples.*—Before collecting milk, each udder was washed with a mild hypochlorite solution using a separate disposable paper towel for each animal. All milk samples were collected in sterile screw-capped 15-ml. vials. Duplicate samples were collected from each quarter; one containing brom-cresol purple was used for bacteriological studies and one without the dye was used for the modified Whiteside test, virus recovery, and neutralization studies. The sample for bacteriological study was placed in an incubator set at 37 C. and the other sample was placed in a refrigerator at 4 C. for 24 hours.

Milk samples were collected once every 24 hours for the first seven postinoculation days, once every 48 hours for the next six days, and then once weekly up to one month postinoculation. After the small amount of milk was withdrawn from the vials without the dye for the modified Whiteside tests, the milk was frozen and held at −20 C. until the virus recovery and neutralization tests could be performed.

* Isolated by F. R. Beaudette, New Jersey Agricultural Experiment Station, New Brunswick, April, 1956.

*Bacteriological Examination of the Milk.*—All milk samples collected in this study were cultured and the various types of bacteria were determined. This was to determine whether any changes occurred in the bacterial flora of the milk as a result of introducing the various viruses into the udder.

The milk was incubated for 18 to 24 hours at 37 C. and then transferred to blood agar medium and incubated another 18 to 24 hours at 37 C. The bacteria were grouped as streptococci, staphylococci, bacilli and coliforms, and micrococci. If staphylococci were present, the coagulase test was performed.

*Whiteside Test and Leukocyte Counts.*—The modified Whiteside test (MWT)[20] and leukocyte counts[18] were used in this study as an index of inflammatory response in the udders due to the introduction of the viral agents. A 1+ MWT or a count of 500,000 cells per milliliter, or both, was considered indicative of inflammation in the udder.

*Virus Recovery from Mammary Glands and Blood.*—Attempts were made to recover virus from the milk and blood at intervals after it was inoculated into the mammary gland. All milk or blood tested for presence of virus was inoculated into embryonating eggs for primary isolation.

Fluids from eggs in which the embryos died after the inoculation of milk were tested as follows: Those samples expected to contain VAC were inoculated intradermally in rabbits and observations made for the appearance of typical skin pocks. Fluids expected to contain VSV were inoculated intracerebrally into mice and observations were made for central nervous system disturbances and death. Egg fluids expected to contain NDV were tested for hemagglutination activity. All egg fluids were tested on nutrient agar and thioglycollate broth for bacterial sterility.

*Virus Neutralization Tests.*—The development of specific antibodies in the serum and milk following the introduction of the three viruses into the mammary glands was determined by employing the virus neutralization test. The method of a constant quantity of antibody (serum or milk) and a varying quantity of seed virus was used. All serum and milk samples, except those tested for vaccinia antibodies, were inactivated at 56 C. for 30 minutes. The vesicular stomatitis and Newcastle disease antibody-virus mixtures were allowed to incubate at 4 C. for a minimum of 30 minutes. The vaccinia antibody-virus mixtures were incubated for a minimum of 60 minutes at 4 C. The infectious titers of all serum or milk-virus mixtures were determined in embryonating eggs.

## RESULTS

The body temperature responses to the intramammary infusion of VAC and NDV were recorded at 24-hour intervals (table 1). The temperatures of cows inoculated with VSV were checked more frequently. Elevated temperatures (104.4–106.2 F.) were recorded in 5 of 6 cows inoculated

**TABLE 1—Response of 17 Cows to the Instillation of a Virus into the Mammary Gland**

| Animal and treatment | Condition | Systemic reaction* | Udder swelling | Modified Whiteside test† | Leukocytes** | Bacterial changes‡ | Virus isolation from milk§ |
|---|---|---|---|---|---|---|---|
| Vaccinia | | | | | | | |
| 279 | L | 0 | + | 3 | 3 | 0 | + |
| 2 | L | 2 | + | 13 | 12 | 0 | + |
| 9 | L | 1 | + | 9 | 8 | 0 | + |
| 51 | L | 1 | + | 6 | 4 | 0 | + |
| 36 | NL | 2 | + | .... | .... | .... | .... |
| 47 | NL | 2 | — | .... | .... | .... | .... |
| Newcastle disease virus | | | | | | | |
| 52 | L | 0 | — | 28 | 21 | 0 | + |
| 281 | L | 1 | — | 6 | 6 | 0 | + |
| 237 | NL | 0 | — | .... | .... | .... | .... |
| Vesicular stomatitis virus | | | | | | | |
| 16 | L | 1 | — | 4 | 2 | 0 | + |
| 20 | L | 1 | — | 3 | 3 | 0 | + |
| 31 | L | 2 | — | 4 | 3 | 0 | + |
| 235 | L | 0 | — | 2 | 2 | 0 | — |
| 44 | L | 1 | — | 4 | 3 | 0 | + |
| 237 | NL | 1 | — | .... | .... | .... | .... |
| 240 | NL | 0 | — | .... | .... | .... | .... |
| 241 | NL | 0 | — | .... | .... | .... | .... |
| Normal | | | | | | | |
| 18 | L | 0 | — | 3 | 3 | 0 | — |
| 99 | L | 0 | — | 2 | 2 | 0 | — |

L = lactating; NL = nonlactating. * No. of days on which a temperature of 103.5 or higher was observed within the first six days postexposure. † No. of consecutive days postexposure that milk gave a 1+ reaction. ** No. consecutive days that milk contained 500,000 or more cells per milliliter. ‡ 0 = no significant increase or decrease in normal flora or the appearance of other types. § Isolation from milk on one or more occasions one to nine days postexposure (+) or no isolation (—).

with VAC. This temperature elevation occurred 48 to 96 hours postinoculation. One of 3 cows inoculated with NDV had an elevated temperature (104.8 F.) 24 hours after inoculation. Elevated temperatures (103.5–106.2 F.) were recorded in 5 of 8 cows inoculated with VSV. No elevated temperatures were observed in the control cows after the inoculation of noninfected allantoic fluid or chorioallantoic membrane suspension. There were no other systemic physical signs attributable to the intramammary infusion of these agents.

No local abnormalities were observed or palpated in any of the cows inoculated with VSV. In the animals given VAC, half of the inoculated quarters were swollen and tender. Lesions appeared on the ends of all inoculated teats and were confined to this area with no spreading upward or to uninoculated teats. The teat lesion progressed from a papule to a vesicle to a scab and then healed in 14 to 21 days. One nonlactating cow showed an extensive swelling of the infused quarters that began 48 hours postinoculation. This udder seemed abnormal, to physical examination, until 15 days after infusion. No lesions were noticed on the ends of the infused teats. The udder swelling extended well up the es-

**TABLE 2—Leukocyte Counts and Modified Whiteside Test (MWT) Reaction in a Cow Inoculated with $3 \times 10^{7.16}$ e.l.d.$_{50}$ (Yolk Sac) of Vaccinia Virus per Quarter**

| | Uninoculated quarter | | | | | Inoculated quarter | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Date | MWT | Total No. of cells | Granulocytes | Agranulocytes | Epithelial cells | MWT | Total No. of cells | Granulocytes | Agranulocytes | Epithelial cells |
| 17 April | — | 0.03 | 0.03 | 0.00 | 0.0 | — | 0.0 | 0.0 | 0.0 | 0.0 |
| 18 April | — | 0.00 | 0.00 | 0.00 | 0.0 | — | 0.0 | 0.0 | 0.0 | 0.0 |
| 20 April | — | 0.03 | 0.00 | 0.03 | 0.0 | — | 0.15 | 0.0 | 0.15 | 0.0 |
| 23 April* | — | 0.14 | 0.04 | 0.10 | 0.0 | 3+ | 28.2 | 22.8 | 5.6 | 0.0 |
| 24 April | — | 0.00 | 0.00 | 0.00 | 0.0 | 2+ | 10.8 | 8.8 | 2.0 | 0.0 |
| 25 April | — | 0.00 | 0.00 | 0.00 | 0.0 | 3+ | 9.7 | 5.2 | 4.5 | 0.0 |
| 26 April | — | 0.05 | 0.00 | 0.05 | 0.0 | 3+ | 4.8 | 2.4 | 2.4 | 0.0 |
| 27 April | — | 0.08 | 0.00 | 0.08 | 0.0 | 3+ | 19.3 | 9.1 | 10.1 | 0.0 |
| 28 April | — | 0.10 | 0.00 | 0.10 | 0.0 | 3+ | 27.4 | 12.6 | 14.8 | 0.0 |
| 1 May | — | 0.00 | 0.00 | 0.00 | 0.0 | 2+ | 1.8 | 0.43 | 1.1 | 0.22 |
| 3 May | — | 0.16 | 0.02 | 0.13 | 0.0 | 2+ | 1.2 | 0.48 | 0.64 | 0.08 |
| 5 May | — | 0.03 | 0.00 | 0.03 | 0.0 | 1+ | 0.3 | 0.03 | 0.27 | 0.0 |

Numbers indicate average number of cells per oil immersion field (1 cell equal to approximately 500,000 cells per milliliter with microscope used).

* 24 hours postinoculation.

XUM

cutcheon and there was extensive edema along the ventral abdominal wall anterior to the udder.

No clinical abnormalities were noticed in the lactating cows inoculated with NDV. There was, however, a pronounced swelling in the inoculated quarters of a nonlactating animal. The swelling was most severe 48 hours after inoculation but had completely disappeared at 96 hours.

No clinical abnormalities were observed in the control cows. The herdsman noticed a drop in milk production in all the animals after the udder infusion. However, records were not kept regarding the severity of the drop or how long it persisted.

All quarters that were infused showed some degree of inflammation as determined by the MWT and leukocyte counts. The data (table 2) indicate the general trend of MWT reactions and leukocyte counts following the infusion of the various agents into the udder. Leukocyte counts of at least $17 \times 10^6$ cells per milliliter of milk were recorded. The response to the VAC and NDV was generally more severe and lasted longer than the response to VSV (table 1).

No change was detected in the bacterial flora of any quarter during this study. The bacterial counts from some quarters appeared to drop sharply 24 hours after inoculation and return to their previous level by 48 hours. This was presumably due to the sudden increase of leukocytes. (This was a casual observation, not an exact count.)

The specific viruses were recovered from the milk of the inoculated lactating quarters at varying times after infusion. No virus was demonstrated in uninoculated quarters. The VSV was recovered from eight of the ten lactating quarters that were inoculated with this virus. This virus was demonstrated at 24 hours but not 48 hours after inoculation. The VAC was recovered from all eight of the inoculated lactating quarters. In 3 of the cows, the virus was demonstrated at six but not at nine days after inoculation; in the fourth cow, virus was demonstrated at eight but not at ten days. Newcastle disease virus was recovered from the four inoculated quarters at six but not nine days after inoculation. No attempt was made to recover any of the viruses from nonlactating quarters. Attempts to demonstrate a viremia with these three agents were not successful.

**TABLE 3—Antibody Response of Cows to Virus Instilled into the Mammary Gland**

| Animal No. | Quarter milk samples A | B | C | D | Serum |
|---|---|---|---|---|---|
| VACCINIA VIRUS | | | | | |
| 279 | 0 | 0* | 0 | 0* | 2 |
| 9 | 10 | 0* | 0 | 0* | 100 |
| 51 | 0* | 0 | 0* | 0 | 191 |
| 36 | .... | NL | .... | .... | 2 |
| NEWCASTLE DISEASE VIRUS | | | | | |
| 52 | .... | 1,120* | 58 | 4,570* | 70,000 |
| 281 | 3,350,000 | 850,000* | 89,100 | 2,240,000* | 7,000,000 |
| 237 | .... | NL | .... | .... | 399 |
| VESICULAR STOMATITIS VIRUS | | | | | |
| 16 | 17,400* | 17,400 | 21,400* | 12,300 | 1,130 |
| 20 | 7,250 | 7,250* | 9,120 | 5,750* | 316 |
| 31 | 1,000 | 398* | 1,580 | 708* | 2,270 |
| 235 | 1,740 | 1,350* | 1,780 | 2,510* | 56,000 |
| 44 | 5,620 | 100,000* | 5,620 | 6,310 | 56,000 |
| 237 | .... | NL | .... | .... | 13,300 |

Numbers are neutralizing indexes, the antilog of the difference between the virus titer ($Log_{10}$) and the antibody-virus titer ($Log_{10}$).
NL = not lactating; * inoculated quarter.

No effort was made to determine when neutralizing antibody appeared in the milk from the various quarters, or in the serum. The amount of neutralizing antibody was first determined in the serum and milk two weeks after intramammary inoculation with VSV and one month after inoculation of VAC and NDV (table 3). The virus-neutralizing ability of the milk from the animals inoculated with VAC was essentially zero, even in those animals that had fairly good serum-neutralizing indexes. The neutralizing ability of milk from the animals inoculated with NDV was comparable to the neutralizing ability of their serums. There was no pattern of antibody level in the quarter samples in relation to the quarters inoculated.

In the process of determining the neutralizing ability of the milk from the quarters inoculated with VSV, it was observed that milk obtained prior to the inoculation was capable of neutralizing up to 5,000 embryo $l.d._{50}$ of VSV (table 4). There was no history to indicate that the animals had been exposed to VSV, and the blood serum had no significant VSV-neutralizing ability prior to inoculation with the VSV (table 5). Every milk sample tested thus far from field herds has shown some VSV-neutralizing ability.

**TABLE 4—Virus Neutralizing Ability of Milk Before and After the Inoculation of Vesicular Stomatitis Virus into the Bovine Mammary Gland**

| Animal No. | Virus control | A quarter | | B quarter | | C quarter | | D quarter | |
|---|---|---|---|---|---|---|---|---|---|
| | | Before | After | Before | After | Before | After | Before | After |
| 16 | 4.8 | 2.5 | 0.6 | 2.6 | 0.6 | 3.0 | 0.5 | 2.1 | 0.7 |
| 20 | 5.3 | 3.6 | 1.5 | 1.7 | 1.5 | 1.6 | 1.4 | 2.4 | 1.6 |
| 31 | 4.6 | 2.6 | 1.6 | 1.6 | 2.0 | 1.6 | 1.4 | 1.6 | 1.7 |
| 44 | 5.5 | 2.5 | 1.7 | 3.0 | 0.5 | 2.7 | 1.7 | 2.7 | 1.7 |
| 235 | 5.0 | 3.4 | 1.7 | 2.6 | 1.8 | 2.2 | 1.7 | 3.0 | 1.6 |

Number indicates $e.l.d._{50}$ titer of vesicular stomatitis virus ($Log_{10}$ per 0.1 ml.) when inoculated without milk (control) or with milk (quarter samples).

## DISCUSSION

In determining whether a virus could produce mastitis when introduced into the mammary gland, it was necessary to ascertain whether an inflammatory reaction, and therefore tissue damage, was present in the udder after introduction of the virus. Because of the nature of this study, *i.e.*, the desirability of determining the period of persistence of the virus in the mammary gland and the production of antibody, it was not practical to slaughter the cows to determine whether histological changes had taken place in the mammary gland. Therefore, indirect methods were necessary to indicate the presence of an inflammatory reaction. The MWT and direct leukocyte counts were selected to indicate the presence of an inflammatory reaction.

If the cows in this study had been encountered in a field herd, the diagnostician would have observed the increased number of leukocytes in the milk and the increased MWT reaction. None of the animals would have been shedding bacteria in their milk that are considered to be udder pathogens. The history would have included swollen and sore udders, sore teats, a drop in milk production, and possibly an elevated body temperature. The diagnostician would have described the cases as idiopathic mastitis on the basis of the MWT, leukocyte counts, and bacteriological cultures.

This study does not substantiate or question the role of viruses in mastitis of dairy cows. It seems quite evident on the basis of our work and that of Mitchell *et al.*[11-15] that a number of viruses can not only propagate within the mammary gland but also can produce inflammation. Some of the idiopathic cases of mastitis encountered by the practitioners may, indeed, be a result of a virus infection of the mammary gland. Whether the viruses, if any, would be specific mammary gland viruses or one or more of any number of known viruses, is still a matter of speculation.

**TABLE 5—Virus Neutralizing Ability of Serum Before and After the Inoculation of Vesicular Stomatitis Virus into the Bovine Mammary Gland**

| Animal No. | Virus | Preinoculation serum | | Postinoculation serum | |
|---|---|---|---|---|---|
| 16 | 6.0 | 5.3 | (0.7) | 2.5 | (3.5) |
| 20 | 6.0 | 5.0 | (1.0) | 2.5 | (3.5) |
| 31 | 6.0 | 4.8 | (1.2) | 1.5 | (4.5) |
| 44 | 5.6 | 5.5 | (0.1) | 0.75 | (5.0) |
| 235 | 5.6 | 4.5 | (1.1) | 0.75 | (5.0) |

Number indicates $e.l.d._{50}$ titer of virus or virus-serum mixture ($log_{10}$ per 0.1 ml.). Numbers in parentheses indicate $log_{10}$ $e.l.d._{50}$ neutralized.

No attempt was made to follow the appearance or disappearance of antibody in this study. Mitchell *et al.*,[11,14,15] who studied the appearance of antibody in milk from infected and uninfected quarters and in the blood serum, thought that antibody is produced first in the infected quarters.

The inactivation of VSV by normal milk and milk components was an unexpected observation in this study. Mitchell[11] reports that "the viruses of eastern, western and Venezuelan equine encephalomyelitis and lymphochoriomeningitis are inactivated within the mammary gland likely by a substance present there." The nature of this inactivation or neutralization is obscure.

XUM

## SUMMARY AND CONCLUSIONS

An inflammatory reaction was produced in the bovine mammary gland by the infusion of vesicular stomatitis virus, Newcastle disease virus, and vaccinia virus through the teat canal into the udder. These viruses persisted in the mammary gland for varying periods and in most cases stimulated the production of considerable neutralizing antibody. Milk from cows not previously exposed to vesicular stomatitis virus contained a heat-stable, vesicular stomatitis virus-inhibiting substance.

The fact that viruses and other nonbacterial agents may induce mastitis in the field should not be overlooked when mastitis diagnostic problems arise.

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# A Study of the Staphylococci Associated with the Bovine Udder

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ALTHOUGH *Staphylococcus aureus* is associated with acute and chronic bovine mastitis, it also may be found in apparently healthy udders. Miller and Heishman [22] have demonstrated that if staphylococci from an acute mastitic condition are introduced into a healthy udder they may cause an acute mastitis, whereas Schalm [24] has shown that staphylococci from an apparently healthy udder may, on injection into normal udders, be responsible for acute mastitis. Thus, it would appear that the severity of a staphylococcic mastitis infection may depend on either the high virulence of the infecting organisms or the low resistance of the udder tissue.

As it is possible that the virulence of the organism may vary with different physiological types, the present investigation was carried out to determine whether cultures isolated from animals with cases of acute or chronic mastitis or from apparently normal udders showed any physiological difference *in vitro*. At the same time, it was realized that the presence of disease in the udders from which the cultures were obtained could depend on the resistance of the udder tissues to the organisms.

## MATERIALS AND METHODS

Cultures of *Staph. aureus* which had been isolated from cows with cases of acute mastitis,* chronic mastitis,† and from clinically normal udders† were used. The mastitis was regarded as acute if there was systemic upset, the udder hard, painful and swollen, and the milk grossly abnormal; it was considered to be chronic if the disease was milder but persistent, with leukocyte counts of the milk exceeding 1,000,000 per milliliter.

The following characters were determined for each culture by means of the standard methods: hemolysis on bovine blood agar; coagulase production; fermentation of glucose, sucrose, lactose, mannitol, glycerol, raffinose, inulin, salicin, xylose, arabinose, and sorbitol; production of hydrogen sulfide; and action on litmus milk.

Pigment and gelatinase production were determined at 25 C., 37 C., and 42 C., using the medium [10] devised by Chapman [6] for the Stone gelatinase test. For lipase production, neutral red agar plates were employed in duplicate, one containing 0.4 per cent bovine fat and the other 0.4 per cent tristearin. Tolerance of sodium chloride was determined in nutrient agar for concentrations of the salt increasing by 0.5 per cent amounts from 10.0 to 14.0 per cent. Urease production was tested in Bacto-urea* broth.[10] The antibiotic sensitivity for penicillin, dihydrostreptomycin, oxytetracycline (Terramycin†) and chlortetracycline (Aureomycin**) was examined by means of sensitivity disks;‡ the vitamin requirements by the method of Gretler *et al.*; [15] the opacity in egg yolk medium according to the method of Gillespie and Alder; [17] and the bacteriophage typing by Seto's [25] modification of the methods of Jackson *et al.*,[20] Blair and Carr,[3] and Williams and Rippon.[31]

In the determination of exotoxin production, the type of lysin formed was identified by the method of Elek and Levy; [12] *alpha-* and *beta*-lysins were produced and titrated according to the procedure described by Elek and Levy.[13] The presence of dermonecrotizing toxin was shown by the necrotizing effect of a subcutaneous injection of 0.1 ml. of the toxin into each of 2 white rabbits. The lethal effect was examined by injecting 0.5 ml. of the toxin into the tail vein of each of 3 mice, and the time which elapsed until death was recorded.

## RESULTS

Seventy cultures of *Staph. aureus* were examined; 15 of these were from cows with acute mastitis, 29 from cows with chronic mastitis, and 26 from normal udders.

With the exception of one of the cultures from a cow with chronic mastitis, all were coagulase positive. All these coagulase-positive strains required in a casein hydrolysate medium the vitamins thiamin and nicotinic acid, and most showed a slight to marked increase of growth in the presence

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* Cultures from Dr. R. A. Packer, Iowa State College, Ames; Dr. G. R. Spencer, Washington State College, Pullman; and Dr. H. L. Shute, University of Maine, Orono.

† Cultures from Dr. J. Simon, University of Wisconsin, Madison.

* Bacto-urea is produced by Difco Laboratories, Detroit, Mich.

† Terramycin is produced by Chas. Pfizer & Co., Brooklyn, N.Y.

** Aureomycin is produced by Lederle Laboratories, Division of American Cyanamid Co., New York, N.Y.

‡ Sensitivity disks are produced by Difco Laboratories, Detroit, Mich.



XUM

TABLE 1—Acid Production and Action on Various Substrates of Staphylococci Isolated from Udders with Mastitis and from Normal Udders

| Source | Per cent producing acidity | | | | | | | | | | Per cent producing effect on litmus milk | | | Total No. of cultures |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Sucrose | Lactose | Mannitol | Glycerol | Raffinose | Inulin | Salicin | Sorbitol | Xylose | Arabinose | Acid | Reduction | Peptonization | |
| Acute | 100.0 | 100.0 | 100.0 | 100.0 | 93.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.0 | 53.3 | 26.7 | 15 |
| Chronic | 96.5 | 93.1 | 100.0 | 89.7 | 69.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.9 | 6.9 | 17.2 | 72.5 | 6.9 | 29 |
| Normal | 96.1 | 80.8 | 92.3 | 73.1 | 69.2 | 0.0 | 0.0 | 0.0 | 0.0 | 3.9 | 3.9 | 26.9 | 53.9 | 3.9 | 26 |

of biotin. The coagulase-negative culture failed to grow in the casein hydrolysate medium even in the presence of thiamin, nicotinic acid, biotin, riboflavin, pyridoxine, pantothenic acid, and folic acid, and so differed from the 46 coagulase-negative cultures examined by Gretler *et al.*[18] All the cultures were sensitive to penicillin, dihydrostreptomycin, chlortetracycline, and oxytetracycline. All cultures showed about the same tolerance for sodium chloride, the average tolerance of the culture from acute mastitis, chronic mastitis, and normal udders being 11.03 per cent, 11.1 per cent, and 11.65 per cent, respectively.

The fermentation reactions and action on litmus milk are given (table 1). None of the cultures produced hydrogen sulfide or utilized tristearin, so these reactions have not been included in the tables. The staphylococci associated with acute and chronic mastitis could not be differentiated from those isolated from normal udders by their fermentative reaction. With regard to the action in litmus milk, 26.7 per cent of the staphylococci isolated from udders with acute mastitis were proteolytic as against 6.9 per cent from udders with chronic mastitis and 3.9 per cent from normal udders.

The cultures from acute mastitis showed a higher incidence of pigment production, mannitol fermentation, gelatinase production, and lipolysis of bovine fat than did the cultures from the other sources (table 2). The temperature of incubation was not found to influence pigment production. Opacity in egg yolk medium was produced by 73.3 per cent of the acute mastitis strains, but only by 6.9 per cent and 7.7 per cent of the cultures from chronic mastitis and normal udders, respectively.

Two of the cultures from udders with chronic mastitis and one from a normal udder failed to hemolyze bovine blood when grown in air or in $CO_2$. Two cultures from udders with chronic mastitis and two from normal udders were hemolytic only in $CO_2$. The nonhemolytic strains could not be differentiated from the hemolytic by other characters; *e.g.*, pigment production, action on mannitol, or phage typing. The most common hemolytic pattern of the cultures associated with acute and chronic mastitis was *alpha-beta-delta* (table 3), a pattern not so frequent in the cultures from normal udders. All the strains isolated from udders with acute mastitis contained

*alpha*-lysin (table 4), compared with 65.6 per cent from udders with chronic mastitis and 73.1 per cent from normal udders. The acute strains also showed a slightly higher incidence of *delta*-lysin. Strains producing *alpha*- or *beta*-lysin showed no difference in the amount of lysin produced, regardless of the source of the culture (table 5).

A greater number of strains were phage typeable from acute mastitis than from the other two sources. The strains isolated from udders with acute and chronic mastitis were more frequently sensitive to phages of the miscellaneous group than to those of the other groups or combinations of groups (table 8). The most common phage types were 44A and 42D (table 9), the incidence of the latter being higher than 42D in the cultures from udders with acute and chronic mastitis. Among the egg yolk-positive cultures there was a higher incidence of strains sensitive to phage types of groups I and II than among the egg yolk-negative cultures (table 10).

**TABLE 2—A Comparison of the Production of Pigment, Mannitol Fermentation, Gelatinase, Lipase, Urease, and Opacity, in the Egg Yolk Medium of Staphylococci from Bovine Udders**

| | | Per cent producing positive reaction | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pigment | | | | | | | | |
| Source | Total No. of cultures | Orange | Yellow | Cream | White | Mannitol | Gelatinase | Urease | Lipolysis of bovine fat | Egg yolk |
| Acute | 15 | 73.3 | 27.7 | 0.0 | 0.0 | 100.0 | 86.7 | 0.0 | 93.3 | 73.3 |
| Chronic | 29 | 31.0 | 52.0 | 13.9 | 3.5 | 89.7 | 65.5 | 6.9 | 38.0 | 6.9 |
| Normal | 26 | 26.9 | 30.8 | 26.9 | 15.4 | 73.1 | 66.1 | 7.7 | 61.5 | 7.7 |

*Alpha*-lysin was present in all the egg yolk-positive cultures (table 6), but in only 66.1 per cent of the egg yolk-negative cultures. *Delta*-lysin was also more frequently produced by the egg yolk-positive cultures. There was a complete correlation between the production of *alpha*-lysin and of the lethal and dermonecrotizing toxin (table 7). With one exception, all the strains which produced toxin, killing mice in less than two minutes, had an *alpha* titer of 1:1,000 or higher. The toxins of strains of a lower *alpha* titer took from four minutes to nine hours to have a lethal effect. *Epsilon*-lysin was present only in the coagulase-negative strain. Four cultures were examined by the cat test for enterotoxin production.* Two of these were egg yolk positive, one from an udder with acute mastitis and the other from a normal udder. The other two cultures were egg yolk negative, one being from an animal with chronic mastitis, the second from a normal udder. Enterotoxin was found only in the egg yolk-positive strain from a normal udder.

**TABLE 3—The Hemolytic Pattern of Staphylococci Isolated from the Bovine Udder**

| | No. of strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Source | *Alpha* | *Beta* | *Delta* | *Alpha Beta Delta* | *Alpha Beta* | *Alpha Delta* | *Beta Delta* | *Epsilon* | Total No. of cultures |
| Acute | 4 | 0 | 0 | 6 | 1 | 4 | 0 | 0 | 15 |
| Chronic | 0 | 7 | 2 | 9 | 5 | 4 | 1 | 1* | 29 |
| Normal | 6 | 0 | 2 | 4 | 6 | 2 | 5 | 0 | 26 |
| Total | 10 | 7 | 4 | 19 | 12 | 10 | 6 | 1 | 70 |

* Coagulase negative culture.

## DISCUSSION

It is commonly accepted that the coagulase-positive staphylococci form a fairly homogeneous group as regards certain characters; *e.g.*, pigmentation, fermentation reactions, and gelatinase production.[14] With regard to pigmentation, all the strains from animals with acute mastitis were found to produce orange or yellow pigment, whereas strains from cows with chronic mastitis or from normal udders showed orange, yellow, cream, or white pigmentation. The fact, however, that more than half (57%) of the cultures from normal udders produced orange or yellow pigment indicates that such pigmentation can not be used as a criterion of pathogenicity. According to Innes,[19] only strains producing *alpha*-lysin are pigmented at 42 C., but

* These tests were performed by Dr. Elizabeth Wilson and Dr. E. Campbell, U.S.P.H.S., Cincinnati, Ohio.

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**TABLE 4—The Percentage Incidence of the Four Types of Hemolysin in Staphylococci from Bovine Udders**

| Source | *Alpha* | *Beta* | *Delta* | *Epsilon* | Total No. cultures |
|---|---|---|---|---|---|
| Acute | 100.0 | 46.7 | 66.7 | 0.0 | 15 |
| Chronic | 65.6 | 75.9 | 58.6 | 3.5* | 29 |
| Normal | 73.1 | 57.7 | 50.0 | 0.0 | 26 |
| Total | 75.7 | 62.9 | 57.1 | 1.4 | 70 |

* Coagulase negative culture.

in this examination, all pigmented strains produced pigment at this or other incubation temperatures, irrespective of the hemolytic pattern.

It has commonly been found [7, 9, 15] that the coagulase-positive staphylococci have similar fermentative capacities. This is confirmed by the present work, but cultures from udders with acute mastitis tended to have wider fermentative powers than those from udders with chronic mastitis or from normal udders. It has been claimed [5, 14] that mannitol fermentation has a closer correlation with pathogenicity than has the fermentation of other carbohydrates, but Smith [27] found that although 95.5 per cent of the pathogenic staphylococci examined by him fermented mannitol, 55.5 per cent of the nonpathogenic staphylococci also did so. Our results agree fairly closely with these findings as, although most of the strains from diseased udders (100% from acute and 89.7% from chronic mastitis) fermented mannitol, 73.1 per cent from normal udders did likewise.

None of the cultures from acute mastitis was able to utilize urea, but this is a property more commonly associated with coagulase-negative strains.[16]

Although the proportion of strains which peptonized milk, produced gelatinase, and lipolysed bovine fat was higher in the cultures from udders with acute mastitis than in those from udders with chronic mastitis or from normal udders, the difference was not so marked as to render these reactions of value as differential criteria. However, with regard to the egg yolk reaction, there was a well-marked differentiation. Thus, 73.3 per cent (12 of 15) of the strains from udders with acute mastitis produced opacity in an egg yolk medium, as against 6.9 per cent (2 of 29) of those from udders with chronic mastitis and 7.7 per cent (2 of 26) from normal udders. This is in agreement with the findings of Alder *et al.*,[1] who, in an examination of human patients, found a higher incidence of egg yolk-positive strains in the cultures isolated from abscesses and others clinical sites than from healthy noses and throats. The clinical sources from which these workers obtained their egg yolk-positive strains would correspond more closely to the conditions existing in the udder with an acute case of bovine mastitis than to an animal with a chronic case. These workers also found that only the egg yolk-positive cultures were able to utilize Arachis oil, and they suggested that the egg yolk reaction might be associated with the production of a lipase active against certain of the higher fatty acids. However, in the present work, it was found that none of the staphylococci examined was able to utilize tristearin, irrespective of their egg yolk reaction.

**TABLE 5—Titers of Alpha and Beta Hemolysins of Staphylococci from Bovine Udders**

| Source | Average titer | | Range | |
|---|---|---|---|---|
| | *Alpha* | *Beta* | *Alpha* | *Beta* |
| Acute | 1:1,064 | 1:728 | 1:32—1:3,000 | 1:32—1:2,000 |
| Chronic | 1:1,062 | 1:1,766 | 1:250–1:2,000 | 1:128–1:3,000 |
| Normal | 1:1,225 | 1:822 | 1:256–1:2,000 | 1:128–1:2,000 |

**TABLE 6—The Percentage Incidence of the Four Types of Hemolysin in Egg Yolk-Positive and Egg Yolk-Negative Organisms**

| Egg yolk reaction | Total No. of cultures | Percentage of strains | | | |
|---|---|---|---|---|---|
| | | *Alpha* | *Beta* | *Delta* | *Epsilon* |
| Positive | 15 | 100.0 | 33.3 | 60.0 | 0.0 |
| Negative | 55 | 66.1 | 66.1 | 50.9 | 1.7 |

There does not appear to be any close relationship between the egg yolk reaction and toxin production or other characters. For example, all the egg yolk-positive strains possessed *alpha*-lysin, but so also did 66.1 per cent of the egg yolk-negative strains (table 6). According to many workers,[4, 12, 23, 27] the presence of *beta*-lysin is an important factor in relation to staphylococcic infections of animals; however, in the present investigation, this lysin was found in only 33.3 per cent of the egg yolk-positive strains and it would appear that,

**TABLE 7—A Comparison of the Combinations of Toxins Occurring in Staphylococci from Bovine Udders**

| Toxin | | | | | Source | | |
|---|---|---|---|---|---|---|---|
| Lethal | Dermonecrotic | *Alpha* | *Beta* | *Delta* | Acute | Chronic | Normal |
| + | + | + | + | + | 2 | 2 | 1 |
| + | + | + | − | + | 3 | 0 | 2 |
| + | + | + | − | − | 3 | 0 | 0 |
| + | + | + | + | − | 0 | 1 | 2 |
| − | − | − | + | + | 0 | 0 | 2 |
| − | − | − | + | − | 0 | 4 | 0 |
| Total No. of cultures examined | | | | | 8 | 7 | 7 |

as suggested by Christie *et al.*,[8] the presence of *alpha*-lysin is of greater significance in the production of disease in animals and man.

The complete correlation found between the presence of *alpha*-lysin and lethal and dermonecrotizing toxin supports the view of Smith [29] that these toxins are identical. The presence of enterotoxin in the culture isolated from an apparently normal udder indicates that enterotoxin-producing strains may be present in market milk.

As has been found by other workers,[2, 11, 28] no differentiation of pathogenic from non-pathogenic strains was possible by means of the sensitivity to the phages at present available. In the present work, phage types of almost all groups were found in the staphylococci, whether associated with mastitic or from normal udders. However, the proportion of strains sensitive to phages of group I and, to a lesser extent, to group III, was greater in the egg yolk-positive strains than in the egg yolk-negative strains (table 10). This is in agreement with the findings of Edwards and Rippon [11] that strains of staphylococci isolated from animals with acute mastitis belonged to phage groups I and III. Moreover, in our work, group I shows a high incidence of *alpha*-lysin (table 11), but group IV types, which also had a high incidence of *alpha*-lysin, were isolated about as frequently from normal udders as from those showing acute mastitis (table 8).

**TABLE 8—The Combination of Phage Groups Occurring in Staphylococci Isolated from Bovine Udders**

| Source | Phage groups | | | | | | | | | | | | Total No. of cultures |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | I,IV | I,M* | II | II,IV,M | III | III,M | III,IV,M | IV | IV,M | M | UT† | |
| Acute | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 1 | 1 | 5 | 1 | 15 |
| Chronic | 1 | 0 | 2 | 0 | 0 | 3 | 2 | 0 | 3 | 3 | 7 | 8 | 26 |
| Normal | 2 | 5 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 4 | 4 | 9 | 29 |
| Total | 5 | 5 | 2 | 1 | 1 | 4 | 4 | 2 | 4 | 8 | 16 | 18 | 70 |

* M = miscellaneous group; † UT = untypeable.

**TABLE 9—The Percentage Incidence of Phage Type 42D and 44A in Staphylococci from the Bovine Udder**

| Source | Phage type | |
|---|---|---|
| | 42D | 44A |
| Acute | 33.3 | 60.0 |
| Chronic | 24.1 | 48.3 |
| Normal | 34.6 | 30.8 |
| Total | 30.0 | 44.3 |

The most frequently occurring phage types were 44A (44.3%) and 42D (30.0%). Type 44A has been reported as being the most common staphylococcic phage type of bovine origin in Wisconsin [26] and Australia,[21] whereas 42D is of more frequent occurrence in England [11, 28] and Canada.[30]

The results of the present investigation show that the egg yolk reaction is a valuable criterion of the virulence of *Staph. aureus*, because our data show that the egg yolk-positive types are usually responsible for the acute form of mastitis; the egg yolk-negative strains, which are of more frequent occurrence in the udder, may cause a less severe form of the disease. It has been suggested [29] that the egg yolk-positive

**TABLE 10—A Comparison of the Occurrence of Phage Groups in Egg Yolk-Positive and Egg Yolk-Negative Cultures**

| Egg yolk reaction | Percentage in each phage group | | | | | | Total No. of cultures |
|---|---|---|---|---|---|---|---|
| | I | II | III | IV | M* | UT† | |
| Positive | 33.3 | 6.7 | 24.6 | 20.0 | 46.6 | 13.3 | 15 |
| Negative | 12.7 | 1.8 | 10.9 | 27.2 | 43.6 | 29.1 | 55 |

* M = miscellaneous group; † UT = untypeable.

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**TABLE 11—The Relationship Between the Phage Group and the Hemolytic Pattern of Staphylococci from Bovine Udders**

| Phage group | No. of cultures | Percentage of cultures containing toxin | | | |
|---|---|---|---|---|---|
| | | *Alpha* | *Beta* | *Delta* | *Epsilon* |
| I | 12 | 91.7 | 58.3 | 33.3 | 0.0 |
| II | 2 | 50.0 | 50.0 | 50.0 | 0.0 |
| III | 10 | 70.0 | 30.0 | 60.0 | 0.0 |
| IV | 20 | 90.0 | 45.0 | 40.0 | 0.0 |
| M* | 33 | 69.7 | 57.6 | 51.5 | 0.0 |
| UT† | 18 | 44.5 | 50.5 | 44.4 | 5.6 |
| Total | 95 | 71.6 | 50.5 | 46.3 | 1.1 |

* M = miscellaneous; † UT = untypeable.

strains are of human origin, and this is supported by our findings that such strains have a lower incidence of *beta*-lysin and a greater sensitivity to phage lysis. If this is correct, the acute form of mastitis may be caused by organisms of human origin.

## SUMMARY

The physiological characters of strains of *Staphylococcus aureus* from animals with acute mastitis, chronic mastitis, and from clinically normal udders were examined. There was no significant difference between the cultures obtained from udders with chronic mastitis and those from normal udders, although the latter showed slightly less biochemical activity. However, the strains isolated from cows with acute mastitis were more frequently pigmented and had, as a rule, a higher capacity for peptonization of milk and lipolytic changes than the strains from animals with chronic mastitis or from normal udders. Moreover, 73.3 per cent of the cultures from udders with acute mastitis produced opacity in egg yolk medium, compared with 6.9 per cent of the cultures from udders with chronic mastitis and 7.7 per cent of those from normal udders.

All the cultures from udders with acute mastitis and all those which were egg yolk-positive produced *alpha*-lysin, but in no greater quantities than did the other *alpha*-lysin-producing strains.

The cultures from animals with acute mastitis showed the greatest sensitivity to phage lysis, but no particular phage group was of more frequent occurrence in the acute mastitis strains than in those from the other sources. The egg yolk-positive strains showed a greater sensitivity to the phage types of group I than did the egg yolk-negative strains.

This investigation shows that the strains of *Staphylococcus aureus* associated with the bovine udder can be differentiated into two types: (1) the egg yolk-positive type, which is associated with the acute form of mastitis, and (2) the egg yolk-negative type, which is found in the udder with chronic mastitis or in the clinically normal udder.

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XUM

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# Staphylococcic Phages. I. Lysogenicity in *Staphylococcus Aureus* of Animal Origin

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LYSOGENICITY in *Staphylococcus aureus* of human origin has been established by Fisk,[4] Rountree,[7] and Gorrill.[6] While lysogenicity has also been described for cultures of animal origin (Barnum and Fuller [1] and Seto [8]), there has been no account of the frequency of lysogenicity in such isolates. As a preliminary step in establishing phage typing methods for *Staph. aureus* of animal origin, a detailed study of the extent of lysogenicity in 85 cultures was undertaken.

## MATERIALS AND METHODS

The detection of phage released by lysogenic cultures was performed by assaying filtrates from 24-hour broth cultures that had been incubated at 37 C. One drop of each filtrate was placed on the surface of a Petri dish containing a base layer of tryptose-dextrose agar on which had been poured 4 ml. of soft (0.8%) agar seeded with 1 ml. of a six-hour broth culture of the indicator strain to be tested. After incubation of plates at 37 C. for 18 hours, the presence or absence of phage was noted by the appearance of plaques or areas of clearing where the filtrate had been placed.

All filtrates producing positive results on one or more isolates of *Staph. aureus* were tested for their ability to produce plaques by plating dilutions of the filtrate with a known sensitive host culture.

Eighty-five filtrates prepared as described were tested for the presence of phage by plating them on 100 randomly selected strains of *Staph. aureus* isolated from animal sources. All filtrates were also tested on the culture of *Staph. aureus* from which they were isolated.

## RESULTS

The results obtained are summarized (table 1). All 85 of the filtrates tested were found to contain substances capable of lysing two or more of the 100 cultures against which they were tested. Since the clear zones recorded as positive gave only presumptive evidence of the presence of bacteriophage and may also have been produced by other inhibitory substances (bacteriocidins), all filtrates were diluted and tested for ability to produce plaques. In every instance plaques were demonstrated, indicating that the zones of inhibition were produced by the action of phages.

The term "pattern of lysis" is applied to the set of hosts that a particular phage may lyse. Many of the phages identified produced patterns of lysis on the strains of *Staph. aureus* used, indicating that the 85 filtrates could be classified into several different phage types. On the basis of their action on the 100 strains of *Staph. aureus* and the patterns of lysis produced, these phages could be divided into 13 main classes and 21 miscellaneous classes. The phages in the 13 main classes had similar host ranges; those in the 21 miscellaneous classes were completely different and unrelated to the 13 (*see* Coles [3] for detailed breakdown of phage types). Such results showed that as many as 34 different phage types may have been isolated from the 85 cultures of *Staph. aureus*.

All of the bacterial cultures of *Staph. aureus* isolated from dogs, cats, and horses were completely resistant to the phages isolated from the staphylococci of bovine origin as well as to those isolated from canine, feline, and equine animals.

No correlation could be established between the number of filtrates lysing a culture and the activity of the phages isolated from these strains. Some strains were completely resistant to all bacteriophage activity, *i.e.*, they were lysed by none of the filtrates. However, the filtrates prepared from these same strains were capable of producing lysis on other host strains. Cultures relatively susceptible to the action of other phages were also capable of producing an active phage. Thus, it seems that little correlation can be made between the lysogenic state of strains of *Staph. aureus* of animal origin and the susceptibility to phage types other than the one that it carries.

Although 100 isolates of *Staph. aureus* of animal origin were used, only 60 were lysed by any of the phages.

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## Discussion

In this work, 100 per cent of the cultures tested for the presence of bacteriophage were found to be lysogenic. This high incidence of phage presence may be accounted for by two possible factors. First, a large series (100 isolates) of *Staph. aureus* was utilized as indicators of phage activity, giving a higher probability of detecting phage that might be present. Some of the phages isolated lysed only a limited number of cultures and the presence of these phages could not have been detected if only a limited number of test strains were utilized. A second factor might be that all of the Staphylococcus strains used as a source of phage were selected because they were hemolytic and coagulase positive. Similar results were reported by Fisk [5] and Rountree,[7] who stated that all of the lysogenic strains of *Staph. aureus* were coagulase positive and hemolytic. Such results suggest that lysogenesis, hemolysis, and coagulase activity might be closely linked genetically. Thus, by selecting hemolytic, coagulase-positive cultures, there may be an automatic selection for lysogenic strains.

Of the cultures tested, 40 per cent were completely resistant to the action of any one of the phages isolated from the lysogenic cultures. It is difficult to account for this high percentage of resistant cultures, unless this resistance was the result of immunity due to the prophages residing in the hosts. Bertani [2] pointed out that lysogenic strains are immune to superinfection with the same phage as is carried as prophage. This places immunity to phage infection in a new perspective as a necessary consequence of the establishment and maintenance of the lysogenic condition. It is also known that most of the mutants of a temperate phage have the same immunity specificity. Therefore, if the temperate phages isolated in this study were mutants to an original temperate phage, this immune state might still exist.

Seven of the strains of *Staph. aureus* not lysed by any of the phages were of canine origin, one of equine origin, and one of feline origin. The remaining resistant strains were of bovine origin. Twenty-nine of the resistant strains of *Staph. aureus* of bovine origin were isolated from the same herd of cattle, and the other two cultures were both isolated from a different herd in the same geographic area. It seems plausible that these strains of staphylococci may have been carrying either the same bacteriophage in a lysogenic state or were lysogenized by a mutant strain of the bacteriophage which rendered them immune to the action of the phages isolated.

**TABLE 1—Activity of Filtrates Prepared from Staphylococci of Animal Origin Against Cultures of Staphylococcus Aureus of Animal Origin***

| Filtrate | Total cultures lysed | Filtrate | Total cultures lysed | Filtrate | Total cultures lysed |
|---|---|---|---|---|---|
| F 2 | 19 | F41 | 9 | F 82 | 14 |
| F 3 | 19 | F42 | 16 | F 83 | 16 |
| F 5 | 14 | F43 | 10 | F 84 | 14 |
| F 6 | 18 | F44 | 12 | F 85 | 16 |
| F 9 | 12 | F45 | 11 | F 86 | 14 |
| F10 | 16 | F48 | 15 | F 87 | 11 |
| F11 | 6 | F49 | 12 | F 88 | 8 |
| F12 | 15 | F50 | 12 | F 89 | 12 |
| F13 | 7 | F51 | 11 | F 90 | 7 |
| F14 | 17 | F52 | 9 | F 95 | 7 |
| F15 | 21 | F54 | 6 | F 98 | 7 |
| F17 | 23 | F57 | 13 | F101 | 8 |
| F18 | 7 | F58 | 2 | F102 | 31 |
| F20 | 15 | F59 | 10 | F103 | 31 |
| F21 | 15 | F61 | 7 | F104 | 15 |
| F22 | 9 | F63 | 4 | F109 | 9 |
| F24 | 20 | F64 | 5 | F110 | 5 |
| F25 | 21 | F65 | 19 | F111 | 18 |
| F26 | 24 | F66 | 4 | F112 | 18 |
| F27 | 23 | F67 | 4 | F113 | 14 |
| F28 | 25 | F69 | 19 | F115 | 9 |
| F31 | 24 | F71 | 19 | F116 | 15 |
| F32 | 28 | F72 | 5 | F119 | 11 |
| F33 | 33 | F74 | 4 | F121 | 11 |
| F34 | 20 | F75 | 3 | F124 | 11 |
| F36 | 11 | F79 | 6 | F126 | 7 |
| F39 | 9 | F80 | 9 | F129 | 13 |
| F40 | 8 | F81 | 9 | F131 | 13 |
| | | | | F132 | 13 |

* A complete set of data of the action of each of the 85 filtrates on 100 indicator hosts may be found in the work of Coles.[3]

It is obvious that lysogenization did not render cells immune to all phages, although they are apparently immune to their own phage. There is the possibility that immunity could be due to lysogenization by more than one phage, as was demonstrated by Rountree.[5] Such multiple lysogenization would render a cell immune to several phage types. The presence of more than one phage from a single isolate of *Staph. aureus* was not detected in this study.

## Summary

1) Of 85 isolates of *Staphylococcus aureus* of animal origin, 100 per cent were found to be lysogenic.

2) When filtrates from each of the 85 cultures were tested against each of 100 isolates of *Staph. aureus*, it was found that the phages in the filtrates differed widely

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in their host range, *i.e.*, each phage had a specific "pattern of lysis."

3) The data indicate that immunity of the bacterial host to infection with a particular phage may be determined by the phage with which the host is lysogenized.

4) Resistance or susceptibility to phage lysis depended on the animal from which the Staphylococcus was isolated.

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# Staphylococcic Phages. II. The Use of Human Typing Phages and Adapted Human Typing Phages in the Typing of *Staphylococcus Aureus* of Animal Origin

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PHAGE TYPING of *Staphylococcus aureus* cultures of human origin has become routine in many institutions where infections with this organism are a problem. The Subcommittee on Bacteriophage Typing of Staphylococci, of the International Committee on Bacteriological Nomenclature, London, England, has established a basic series of phages that are divided into four groups as follows: group I, phages 29, 52, 52A, and 79; group II, phages 3A, 3B, 3C, and 55; group III, phages 6, 7, 42E, 47, 53, 54, 70, 73, 75, and 77; and group IV, phage 42D.

As for staphylococci of animal origin, a valuable contribution was made by Smith,[8,9] who used the Wilson series[11] of phages in an epizootic investigation of animal infections. He found that the staphylococci of bovine origin were susceptible to phage 42D, which is an adapted phage active against staphylococci of human origin.[5] Smith subdivided 16 cultures, which were sensitive to phage 42D, into nine different strains with the aid of seven phages isolated from bovine cultures.

The first reported use of "adapted" staphylococcic phages in typing of bovine infections was made by Seto,[6] who used five adapted types (*see* Craigie and Yen[3] for description of "adapted" phages as used in *Salmonella typhosa* typing).

The present study consists of (1) extensive screening of available human typing phages for their ability to lyse *Staph. aureus* of animal origin, and (2) an evaluation of the suitability of these phages and those adapted to animal staphylococci for routine typing of cultures derived from animals.

Contribution No. 169, from the Department of Veterinary Medicine, and contribution No. 343, from the Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan.

Presented in part by the senior author as partial fulfillment of the requirements for the Ph.D. degree in bacteriology at Kansas State College.

## MATERIALS AND METHODS

Twenty-four human typing staphylococcic phages and the 22 indicator strains were used.* In addition to the phages recommended by the Subcommittee on Bacteriophage Typing of Staphylococci, phages 81, VA4, 47C, and 42B were used.

**TABLE 1—Source and Identification of Adapted Human Staphylococcus Phages**

| Human phage (Blair designation) | Original propagating host | Secondary propagating host* | Identification No. of adapted phage |
|---|---|---|---|
| 79 | 79/52A | H74 | A5 |
| 52A | 79/52A | H74 | A6 |
| 47C | 42B/47C | H8 | A7 |
| 42D | 42D | H8 | A8 |
| 42E | 42E | H8 | A9 |
| 42B | 42B/47C | H8 | A11 |
| 7 | 7 | H8 | A10 |
| 81 | 81 | H89 | A13 |

* H stands for bacterial host culture. For complete codification of bacterial and phage materials, *see* Coles.[2]

All human typing staphylococcic phages were propagated by a method described by Swanstrom and Adams.[10] The secondary host for the propagation of "adapted" phages was selected on the basis of the ability of the phage to lyse cultures of staphylococci of animal origin. The designations of the secondary hosts used for adaptation are listed (table 1).

## RESULTS

Of 173 cultures of *Staph. aureus* of animal origin, 86 (49.7%) were resistant to the action of all human typing phages. The results obtained with the human typing phages on the 87 cultures that were susceptible to one or more phages are presented (table 2).

Forty-six (26.6%) of the cultures were lysed by 42D only, and 28 (16.2%) were lysed by both 42D and 81. Thirteen of the cultures (7.5%) were susceptible to one or more of the human typing phages.

Grouping of the cultures according to the human system is difficult, with the exception of the 46 strains that were lysed

* Supplied by Dr. J. E. Blair, Hospital for Joint Diseases, New York, N.Y.



XUM

by 42D only. These isolates are definitely in group IV. Cultures susceptible to both 42D and 81 can not be grouped. However, since 16.2 per cent of all cultures tested were lysed by these two phages, it would lead one to believe than 81, insofar as animal staphylococci are concerned, is more closely related to group IV than to any other group.

A few of the cultures lysed by miscellaneous numbers of the human typing phages may also be placed into groups. Two cultures belong in group I and two in the miscellaneous group.

It is interesting that all of the cultures of *Staph. aureus* of canine or feline origin were resistant to all human typing phages, while the one culture of equine origin was susceptible to 11 of the human typing phages.

The eight adapted phages were tested on 168 of the 173 isolates of *Staph. aureus* of animal origin that had previously been used for testing the human typing phages. The total number of cultures lysed by the adapted phages are shown (table 3).

**TABLE 2—Susceptibility of Staphylococcus Aureus of Animal Origin to Human Typing Phages**

| Human group | Phage No. | Total cultures lysed |
|---|---|---|
| I | 29 | 2 |
| I | 52 | 4 |
| I | 52A | 3 |
| I | 79 | 5 |
| I | 80 | 0 |
| II | 3A | 1 |
| II | 3B | 1 |
| II | 3C | 0 |
| II | 55 | 1 |
| III | 6 | 4 |
| III | 7 | 4 |
| III | 42E | 5 |
| III | 47 | 3 |
| III | 53 | 0 |
| III | 70 | 2 |
| III | 73 | 2 |
| III | 75 | 1 |
| III | 77 | 0 |
| III | 42B | 6 |
| III | 47C | 5 |
| III | VA4 | 0 |
| III | 54 | 0 |
| IV | 42D | 81 |
| Misc. | 81 | 36 |

An increase in the total number of cultures lysed by the adapted phages as compared with the total number of cultures lysed by the original human typing phages was observed in all cases except with phage A13. Adapted phage A13 was capable of producing lysis on fewer cultures than its original phage 81. However, even with the decrease in the total number of cultures lysed, an alteration in the identity of strains lysed was demonstrated. Although many of the strains susceptible to the original phages were also lysed by the adapted phages, a marked difference in the cultures attacked was noticed. This change in the host range, accomplished by the simple expedient of propagating the human typing phages on animal strains of *Staph. aureus*, is probably an example of "host-induced modification."[4]

**TABLE 3—Susceptibility of Staphylococcus Aureus of Animal Origin to Adapted Human Typing Phages**

| Phage | Adapted from | Total cultures lysed |
|---|---|---|
| A 5 | 79 (Group I phage) | 31 |
| A 6 | 52A (Group I phage) | 18 |
| A 7 | 47C (Group III phage) | 45 |
| A 8 | 42D (Group IV phage) | 109 |
| A 9 | 42E (Group III phage) | 42 |
| A10 | 7 (Group III phage) | 78 |
| A11 | 42B (Group III phage) | 25 |
| A13 | 81 (Group Misc. phage) | 27 |

It was possible, with the adapted phages, to subdivide the 168 cultures of *Staph. aureus* of animal origin into eight main groups, 25 definite subgroups, and 13 miscellaneous categories. Thirty-nine patterns of lysis were established using the eight adapted phages.

## DISCUSSION

Previous work has indicated that *Staph. aureus* cultures of animal origin were susceptible to human typing phage 42D.[8] Seto and Wilson[7] found that over half of 102 cultures of bovine origin were lysed by human typing phage 44A. Only 17 cultures in their series were lysed by 42D, and phage 81 was capable of lysing only three of the 102 cultures.

The results obtained in this study do not correlate completely with those obtained by Seto and Wilson,[7] as 86 of the 173 cultures tested were found to be susceptible to the action of phage 42D and 36 to phage 81. This discrepancy in results might be accounted for by the fact that they used diluted samples of phage, while the results reported here were obtained with undiluted human typing phages.

Attempts to place the strains of *Staph. aureus* of animal origin into the broad human groups were not completely successful, as only 50 of the 173 strains were placed into a definite group. The remain-

ing 37 cultures that were lysed by any of the human phages could not be grouped.

Forty-nine per cent of the 173 cultures of *Staph. aureus* of animal origin were resistant to the human typing phages, whereas only 18.8 per cent of these isolates were found to be resistant to the action of the adapted phages. This indicates that the host-induced modification, which apparently occurred, enhanced the ability of these phages to lyse staphylococci of animal origin. Thus, the use of the process of adaptation should be valuable in selecting phages for the routine typing of such cultures.

## SUMMARY

1) *Staphylococcus aureus* strains of animal origin were relatively resistant to phages routinely used in typing cultures of human origin. However, two phage types, 42D and 81, were found to have some ability to produce lysis in animal strains.

2) It was not possible to place all of the staphylococci of animal origin into the broad human groups.

3) Propagation of human typing phages on animal staphylococci results in adaptation of these phages so that they will lyse these hosts. Studies are in progress to determine if this is a process of host-induced modification.

4) While adaptation of human typing phages makes them more usable for the typing of *Staph. aureus* of animal origin, a relatively high percentage of animal staphylococci still remain resistant to the adapted phages.

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# Staphylococcic Phages. III. Typing of *Staphylococcus Aureus* Cultures Isolated from the Bovine Udder

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BOVINE MASTITIS caused by *Staphylococcus aureus* has become more prevalent in the last several years and this organism is now considered to be one of the most important causes of mastitis. Since this microorganism is prevalent in nature, it is difficult to incriminate an isolate of *Staph. aureus* as the cause of a particular case of disease. Epidemiological studies have been hampered by lack of a suitable method to demonstrate the identity of individual isolates. While certain biological characteristics of staphylococci are useful for identification, they are of little value in determining which of several cultures from related sources are identical and which are different.

In human medicine, the problem of differentiating cultures of *Staph. aureus* has been aided by bacteriophage typing. The most important single value of phage typing lies in the ability to identify a set of cultures that may have had a common origin.

Smith,[6, 7] using the Wilson series of phages in an attempt to type animal strains of *Staph. aureus*, found that the staphylococci of bovine origin were susceptible to phage 42D, which is an adapted phage active against staphylococci of human origin.[4] With the aid of seven phages from bovine cultures, he was able to subdivide 16 cultures sensitive to phage 42D into nine different strains.

Barnum and Fuller[1] attempted to type staphylococci associated with bovine mastitis using 42 lysogenic cultures as the phage source. They were able to classify 81.2 per cent of 591 cultures into five groups. Their results indicated that usually an organism belonging to one phage group produced the mastitis in each herd.

Conclusions of Price *et al.*[3] did not agree with those of Barnum and Fuller;[1] they found that in small herds the staphylococci tended to be of a single phage type, while the isolates from a large herd were of various phage types and patterns. Seto and Wilson[5] also reported the isolation of staphylococci of more than one type from 1 cow. An apparent change in phage type occurred in one quarter that had been treated with antibiotics.

Contribution No. 170, Department of Veterinary Medicine, and No. 344, Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan.

Presented in part by the senior author in partial fulfillment of the requirements for the Ph.D. degree in bacteriology at Kansas State College.

This report deals with the typing of *Staph. aureus* cultures isolated from the bovine udder and an evaluation of the results to determine the value of phage typing in epidemiological studies.

## MATERIALS AND METHODS

Using the typing technique previously described, 126 cultures isolated from the bovine udder were subjected to the action of six different phages. These cultures were from six different herds where staphylococcic mastitis was a problem.

Three phages—A8, an "adapted" phage prepared by propagating phage 42D on an animal Staphylococcus; A13, another adapted phage obtained by propagating phage 81 on a strain of *Staph. aureus* of animal origin; and P102, a phage isolated from a lysogenic strain of *Staph. aureus*—were used in typing these 126 cultures. These will be referred to as the "basic" phages. The following groups were designated as a result of this typing procedure: group AnI, those cultures lysed by A8 only; group AnII, those cultures lysed by A8 and P102; group AnIII, those cultures lysed by A8 and A13; group AnIV, those cultures lysed by A8, A13, and P102; and group AnV, those cultures lysed by P102 only.

## RESULTS

Of the 126 cultures tested, 45 isolates were placed in group AnI, 32 in group AnII, 13 in group AnIII, 13 in group AnIV, and six in group AnV; 17 cultures (13.3%) were not lysed by any of the basic phages.

The use of three additional phages—A10, an adapted phage prepared from human phage 7, and P52 and P87, both of which were isolated from lysogenic strains of *Staph. aureus* of animal origin—made it possible to subdivide the strains of *Staph. aureus* into a total of 21 definite patterns of lysis suitable for typing.

The herd distribution of the 126 strains of staphylococci isolated from bovine udders according to the pattern of lysis produced by the three basic phages is tabulated (table 1).

All herds except herd 2 showed the presence of *Staph. aureus* of more than one phage type. The most predominant phage type was A8.

**TABLE 1—Herd Distribution of Patterns of Lysis Using Phages A8, A13, and P102**

| Group | Pattern of lysis | Total cultures lysed | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Herd 1 | Herd 2 | Herd 3 | Herd 4 | Herd 5 | Herd 6 | Total cultures |
| AnI | A8 | 3 | 10 | .. | 6 | 19 | 7 | 45 |
| AnII | A8, P102 | 5 | .... | 8 | .. | .... | 19 | 32 |
| AnIII | A8, A13 | .. | .... | .. | 2 | 5 | 6 | 13 |
| AnIV | A8, A13, P102 | 1 | .... | 1 | 5 | 1 | 5 | 13 |
| AnV | P102 | .. | .... | 2 | .. | 1 | 3 | 6 |

The herd distribution of the 126 strains of staphylococci isolated from bovine udders is shown (table 2) according to the pattern of lysis produced by the three "basic" phages plus the secondary typing phages, A10, P52, and P87.

It appears that a diversity of strains of *Staph. aureus*, as determined by phage typing, may exist within a single herd. The best example of this diversity of types appears in herd 6. Within the 50 cultures from this herd, 16 different patterns of lysis existed. Five patterns of lysis were demonstrated among the isolates from 9 animals in herd 1, three from 11 isolates in herd 2, five from 15 cultures from herd 3, four from 14 cultures in herd 4, and six from 29 cultures in herd 5.

This variation of the phage type existed not only within the herd but also within individual animals. In cow 2 of herd 2, the left forequarter harbored a strain of *Staph. aureus* that was lysed by phages A8, A10, and P87, while the culture isolated from the right forequarter of the same animal was resistant to all phages used. Cow 7 in herd 4 harbored *Staph. aureus* in both forequarters that were lysed by phages A8, A13, P102, A10, P52, and P87, while an isolate of the same organism from the left hindquarter was lysed by phages A8, A13, A10, and P52.

*Staphylococcus aureus* was isolated from all four quarters of cow 12 in herd 5. The isolate from the right hindquarter was lysed by A8, A13, and A10; the culture from the left hindquarter by A8 only; the isolate from the left forequarter by P102, A10, and P87; and the culture from the right forequarter was lysed by A8, A13, and P87. Thus, *Staph. aureus* isolates belonging to four different phage types were demonstrated in this animal.

In 3 cows in herd 6, *Staph. aureus* was recovered from the same quarter on two separate occasions. In two instances, the isolate from the second sample was of the same phage type as the primary isolation. However, in 1 animal (cow 149B), the culture isolated on the first sampling was lysed by phages A8, A13, P102, A10, P52, and P87. The culture isolated from the second

**TABLE 2—Distribution of Staphylococcus Aureus Cultures Isolated from the Bovine Udder When Typed with Phages A8, A13, A10, P102, P87, and P52**

| Pattern of lysis | Herd No. | | | | | | Total cultures lysed by pattern |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| A8, A10 | 2 | 4 | .. | .. | 2 | 1 | 9 |
| A8, A10, P87 | .. | 4 | .. | .. | 8 | .. | 12 |
| A8, A10, P52 | .. | .. | .. | .. | .. | 1 | 1 |
| A8, P87 | 1 | .. | .. | .. | 1 | .. | 2 |
| A8 | .. | 2 | .. | 6 | 8 | 5 | 21 |
| A8, P102, A10 | .. | .. | .. | .. | .. | 1 | 1 |
| A8, P102, P87 | 1 | .. | .. | .. | .. | 1 | 2 |
| A8, P102, A10, P87, P52 | .. | .. | 5 | .. | .. | 10 | 15 |
| A8, P102, P52, P87 | .. | .. | 2 | .. | .. | 1 | 3 |
| A8, P102, A10, P87 | 4 | .. | 1 | .. | .. | 5 | 10 |
| A8, P102 | .. | .. | .. | .. | .. | 1 | 1 |
| A8, A13, A10 | .. | .. | .. | 1 | 4 | .. | 5 |
| A8, A13, A10, P52 | .. | .. | .. | 1 | .. | 4 | 5 |
| A8, A13, A10, P87, P52 | .. | .. | .. | .. | .. | 1 | 1 |
| A8, A13, A10, P87 | .. | .. | .. | 1 | .. | .. | 1 |
| A8, A13, P52 | .. | .. | .. | .. | .. | 1 | 1 |
| A8, A13, A10, P102, P87 | 1 | .. | 1 | .. | 1 | .. | 3 |
| A8, A13, P102, A10, P87, P52 | .. | .. | .. | 5 | .. | 5 | 10 |
| P102, A10, P52, P87 | .. | .. | .. | .. | .. | 1 | 1 |
| P102, A10, P87 | .. | .. | .. | .. | 1 | 1 | 2 |
| P102, P87 | .. | .. | 2 | .. | .. | 1 | 3 |
| None | .. | 1 | 3 | .. | 5 | 8 | 17 |

XUM

sampling two months later was lysed by phages A8 and A10. This change in phage type of the isolated organism represents either a reinfection of the quarter with a different strain of *Staph. aureus* or a change in the pattern of lysis of the organism originally present. It seemed more likely that a reinfection of this quarter occurred.

## DISCUSSION

The results of a series of attempts to type *Staph. aureus* isolated from the bovine udder indicate that a wide variation of phage types may occur within a herd and also within an individual. These results corroborate those of Price *et al.*[3] and Seto and Wilson,[5] who also found a wide variation of phage types of staphylococci that were isolated from a bovine udder. Although Seto and Wilson[5] have reported that the phage types of staphylococci isolated from small herds tend to be of the same type, such results were not obtained in this study. The size of the herd seemed to have little bearing on the number of phage types of staphylococci isolated. The only possible exception was herd 6, in which 18 different phage types were demonstrated. This was the largest herd used in this study, and the results would tend to support those of Seto and Wilson.[5] On the other hand, it was possible in herd 2 to demonstrate four different patterns of lysis in only 5 cows; and in herd 1, five different patterns of lysis were demonstrated from only 9 cows.

Only one strain of *Staph. aureus* was isolated from a single quarter at one sampling. However, 7 cows in herd 6 had udders infected with two or more phage types. In cow 444B, for example, *Staph. aureus* was isolated from all four quarters, and three different phage patterns were demonstrated. Such results indicate that multiple infection of the bovine udder with more than one strain of *Staph. aureus* occurs.

## SUMMARY

1) The two adapted human typing phages, A8 and A13, and phage P102, isolated from a lysogenic strain of *Staphylococcus aureus*, had definite host ranges that might make them usable for epidemiological studies in bovine mastitis.

2) Three additional phages, A10, P52, and P87, had host ranges that were not quite so definite but still might be usable as auxiliary typing phages for epidemiological studies.

3) On the basis of phage typing, evidence is presented to indicate that in most herds of cattle where staphylococcic mastitis is a problem, more than one strain of *Staph. aureus* is present, and a single cow may harbor more than one type of *Staph. aureus* in her udder.

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# Histology of the Urethral Epithelium of Domestic Animals

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LITERATURE on the urethral epithelial lining commonly describes it as transitional in all male domestic animals and as stratified squamous in females. However, in recent extensive work on the urinary system, the author found that the urethral epithelium varied considerably in different species and sexes of domestic animals.

### COMMON DESCRIPTION OF EPITHELIAL LINING

*The Male Urethra.*—Descriptions of the stallion urethral epithelium differ with various authors. Chaveau [4] termed it stratified and cylindrical, McFadyean [14] called it simple columnar, and Bruni [3] stated that it was transitional from the bladder distally as far as the "fossa navicularis" and that beyond this region the epithelium was "pavemental" to the external orifice. Sisson and Grossman [18] observed transitional epithelium in the proximal part, similar to that of the bladder. This changed to cylindrical and became stratified squamous in the terminal part.

Haq [6] found that the transitional epithelium of the pelvic and the extrapelvic parts of the urethra of the bull changed to stratified squamous near the tip of the glans penis. The average thickness of the epithelial coat was 27 to 50 $\mu$ but ranged from 19 to 97 $\mu$. Yao and Eaton [28] described a transitional epithelium six to eight cells deep in the penile urethra of the goat. Strickland-Goodall [19] described a thick stratified urethral epithelium in the vermiform process, glans, and toward the bladder in the ram. The epithelium consisted of six to 12 rows of cells with a definite basement membrane.

McKenzie *et al.*[15] stated that the entire urethra of the boar was lined by pseudostratified low columnar or cuboidal epithelium. The pelvic part showed numerous ducts leading from the glandular tissue to the surface of the urethral lumen, but there was no indication of secretory tissue or activity in the epithelium, either anterior or posterior to the sigmoid flexure.

Christensen [5] referred to a transitional epithelium in the penile urethra of the dog. Strickland-Goodall [19] described, in the tomcat, a thick urethral mucosa resting on a basement membrane and consisting of stratified squamous epithelium "anteriorly." Toward the bladder, the epithelium consisted of three or four cell layers, and the superficial cells were frequently columnar. Langworthy and Kolb [11] described the cat's urethral epithelium as transitional.

Trautmann and Fiebiger,[20] in a comparative study of the domestic animals, stated that the transitional epithelium lining the pelvic urethra in general was also continued in the penile part, where it changed to stratified squamous at the external orifice. The epithelium showed secretory activity in the pelvic part, and in the penile part few glands opened into it.

*The Female Urethra.*—A stratified squamous epithelial lining has been described by most workers (McFadyean,[14] Schmaltz,[17] Sisson and Grossman,[18] and Trautmann and Fiebiger [20]). The epithelium showed tubular evaginations in the mare, cow, and sow. Single, branched, tubular glands also occurred in the cow (Trautmann and Fiebiger [20]). In the mammalian female urethra, Kendall [10] found glandular pockets with mucous cells and deeper sinuses with mucoserous cells. The epithelium rested on a compact connective tissue with elastic fibers, and lymph nodules were also present (Schmaltz,[17] Trautmann and Fiebiger [20]).

The urethral mucosa was longitudinally folded in all animals of both sexes (Chauveau,[4] McFadyean,[14] Sisson and Grossman,[18] Trautmann and Fiebiger [20]). According to Strickland-Goodall [19] and Schmaltz,[17] the layers of the cells and the heights of the epithelium varied greatly in different species of the domestic animals. Martin [13] made some histochemical studies on the transitional epithelial lining of the bladders and ureters of the guinea pig, rabbit, rat, mouse, and cat. Walker [22] made an electron microscopic study of transitional epithelial basement membranes in the frog, mouse, rat, cat, dog, and human being.

### MATERIALS AND METHODS

For this study, 41 animals were used: 3 male and 3 female horses, cattle, sheep, swine, and dogs; 4 male and 3 female cats; and 2 male and 2 female goats.

The entire urethra from the freshly killed animal was fixed in F.A.A. (Lavdowsky's mixture of formalin, alcohol, and acetic acid, Guyer [7]). Following fixation, tissues were taken from three to five levels in the females and eight to 16 levels in the males, depending upon the length of the urethra and the presence of bone, glands, flexures, processes, and diverticulae of the urethrae in different species and sexes. In male dogs and cats, the os penis was decalcified. Dehydration and infiltrating

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**TABLE 1—The Urethral Epithelium of Domestic Animals**

| Species | Epith., cells, height in μ | Male urethra: Penile: Processus urethrae [1] or tip of penis [2] | Male urethra: Penile: Glans penis | Male urethra: Penile: Distal part | Male urethra: Penile: Middle part [1] or sigmoid flexure [2] | Male urethra: Penile: Near bulbus | Male urethra: Pelvic: Near urethral bulb | Male urethra: Pelvic: Middle part | Male urethra: Pelvic: Near bladder | Female urethra: Near external orifice | Female urethra: Middle part | Female urethra: Near bladder | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Equus | E. | [1] Scu, Scl | Scl | Scl | [1] Scl, Tr | Scl, Tr | Tr | Tr | Tr | SS | Scl, Tr | Tr | |
| | C. | 14–20 | 4–14 | 2–8 | 2–7 | 2–10 | 2–9 | 2–9 | 2–9 | 3–16 | 3–8 | 3–14 | Equus |
| | H. | 122–210 | 38–160 | 26–114 | 21–84 | 26–120 | 21–140 | 21–131 | 26–149 | 43–180 | 35–122 | 40–195 | |
| Bovis | E. | Proc. Ur. | SS, Tr | Tr | [2] Tr | Tr | Tr | Tr | Tr | Scl, Tr | Tr | Tr | |
| | C. | embedded | 3–18 | 3–12 | 3–10 | 2–10 | 3–12 | 3–12 | 3–12 | 2–11 | 3–7 | 3–8 | Bovis |
| | H. | in glans | 26–262 | 26–175 | 26–148 | 26–175 | 21–175 | 21–175 | 21–175 | 26–140 | 26–105 | 26–122 | |
| Capra | E. | [1] Tr | Scl, Tr | Scl, Tr | [2] Scl, Tr | Scl, Tr | Scl | Scl, Tr | Tr | SS | Scl | Tr | |
| | C. | 3–6 | 3–5 | 2–5 | 2–5 | 2–5 | 2–4 | 2–5 | 2–5 | 3–10 | 3–8 | 2–6 | Capra |
| | H. | 17–61 | 17–57 | 8–38 | 8–52 | 7–70 | 14–49 | 7–61 | 26–87 | 26–122 | 41–119 | 26–78 | |
| Ovis Young | E. | [1] Tr | SS, Tr | SS | [2] SS | SS | SS, Tr | SS, Tr | Tr | SS | Scl | Tr | Young |
| | C. | 6–12 | 3–13 | 3–10 | 3–11 | 4–12 | 4–11 | 4–13 | 3–12 | 4–15 | 2–6 | 3–10 | |
| | H. | 52–192 | 29–180 | 31–119 | 29–143 | 40–180 | 40–180 | 26–192 | 26–166 | 52–192 | 17–100 | 31–157 | |
| Ovis Adult | E. | [1] Tr | Tr | Tr | [2] Tr | Tr | Tr | Tr | Tr | No difference in the 1 ewe studied | | | Adult |
| | C. | 6–12 | 2–5 | 2–4 | 2–4 | 2–5 | 2–4 | 2–5 | 2–6 | | | | |
| | H. | 52–192 | 10–75 | 26–52 | 35–54 | 21–75 | 17–43 | 17–71 | 24–77 | | | | |
| Suis | E. | [2] SS | Tr | Tr | [2] Tr | Tr | Tr | Tr | Tr | Scu | Tr | Tr | |
| | C. | 3–11 | 2–6 | 2–4 | 2–3 | 2–3 | 2–3 | 2–4 | 2–7 | 2–5 | 2–4 | 3–5 | Suis |
| | H. | 22–126 | 17–73 | 14–49 | 16–49 | 14–35 | 12–31 | 10–38 | 31–94 | 14–63 | 17–52 | 35–87 | |
| Canis | E. | [2] Scl, Tr | Scu, Scl | Scu, Scl | [1] Scu, Scl | Scu, Scl | Scu, Scl | Scl, Tr | Tr | Scu, Scl | Scu, Scl | Scl, Tr | |
| | C. | 4–9 | 2–7 | 2–6 | 1–7 | 2–3 | 2–5 | 2–6 | 3–5 | 2–5 | 2–4 | 2–5 | Canis |
| | H. | 35–110 | 17–78 | 17–78 | 12–64 | 8–47 | 10–52 | 12–80 | 28–105 | 17–66 | 12–61 | 9–66 | |
| Felis | E. | [2] Scl, Tr | Scl, Tr | Scl, Tr | [1] Scl, Tr | Scl, Tr | Scl | Scl, Tr | Tr | SS, Scu | SS, Scu | Tr | |
| | C. | 2–4 | 2–4 | 2–4 | 2–4 | 2–4 | 2–4 | 2–4 | 2–6 | 2–4 | 2–5 | 2–6 | Felis |
| | H. | 8–35 | 10–35 | 10–35 | 10–38 | 8–35 | 10–31 | 7–35 | 12–61 | 7–38 | 8–52 | 12–73 | |

**Abbreviations:** SS = stratified squamous; Scu = stratified cuboidal; Scl = stratified columnar; Tr = transitional; E = epithelium; C = cells; H = height.

**Note:** In the males, the external urinary orifice is lined by stratified squamous epithelium in all the animals and its thickness is about the same as that of the skin of the processus urethrae or the glans. In the females, the height of stratified squamous epithelium at the external orifice has not been considered in calculating the range of height of the urethral epithelium.

methods of Johnson *et al.*[9] were followed, using Tissuemat.*

The following staining techniques were employed: (1) hematoxylin and eosin; (2) Weigert's and Van Gieson's for connective tissue; (3) 0.5 per cent toluidine blue for determining the presence of mucous and mast cells; and (4) azan aniline blue stain was used on some sections to study the nature of certain cells in the epithelium which did not appear to be true epithelial cells.

Epithelial heights were measured at the highest and lowest areas in the epithelium, irrespective of stretching or ballooning of the epithelial cells. The number of the epithelial layers was counted at the typical lowest and highest areas of epithelium.

## Results and Discussion

### General

*The Urethral Epithelium in the Male Animals.*—Penile Urethra.—Apart from being stratified squamous at the external urinary orifice, the type of epithelium and its extension into the penile urethra varied noticeably in different species.

Pelvic Urethra.—The pelvic urethra was lined almost entirely by transitional epithelium except in the buck, dog, cat, and ram lamb. In the ram lamb, the stratified squamous lining extended from the penile part proximally through the pars disseminata, while in the buck, dog, and cat, only stratified columnar epithelium occurred near the bulb, and a mixture of stratified columnar and transitional cells was present in the middle part. Near the bladder, all animals bore transitional epithelium.

*The Urethral Epithelium in the Female Animals.*—Contrary to commonly described stratified squamous epithelium in the urethra of the female domestic animals, a variety of types of epithelium was seen in different animals and at different levels in the same animal, as is the case in the human female.

The stratified squamous epithelium, at the external urethral orifice, was present only in the mare, doe, and ewe and to some extent in the queen (cat). In the other species, the stratified squamous epithelium of the vestibule did not extend into the urethra.

*General Considerations in Both the Sexes.*—The intra-epithelial cysts, mentioned by Maximow and Bloom [13] in man, were quite frequent in the urethral epithelium of every kind in the domestic animals (fig. 10, 19). Fibroblast-like cells were seen extending from the lamina propria into the epithelium of cattle and horses. Their exact nature and function could not be established (fig. 1). "Narrow cells" (Greep [6]) could be seen in the epithelium of most of the animals (fig. 9). The lymphoid aggregation under the epithelium and the migration of lymphocytes through the epithelium were frequent in the urethrae of the domestic animals (fig. 6, 20). This finding differs from the conclusions of Maximow and Bloom,[13] who considered the latter a rare phenomenon. Surface cells with more than one nucleus were seen frequently in the urethral transitional epithelium. The suggestion of Nonidez and Windle [16] that these might represent a stage in cell division could not be confirmed.

In the male animals (except in the ram lambs), the bull had the highest epithelium, followed in decreasing order by stallion, dog, boar, buck, ram, and tomcat; while in the females the mare had the highest epithelium, the other animals in decreasing order being: ewe, cow, goat, sow, queen, and bitch. The height of the epithelium was generally in direct proportion to cell layers in the epithelium. A detailed tabulation of the type of epithelium and various measurements is given (table 1).

### The Horse

*The Stallion.*—Penile Urethra.—The flattened surface cells of the stratified squamous epithelium at the external urethral orifice became high cuboidal, and the epithelium assumed a thick stratified cuboidal or columnar type even distal to the "fossa navicularis." Proximally, the penile urethra had stratified columnar epithelium with occasional patches of transitional epithelium (fig. 1) changing to mostly transitional near the bulbous urethrae. Goblet cells, present in the human cavernous urethra, were absent.

Pelvic Urethra.—In addition to the transitional epithelium, structures similar to the lacunae of Morgagni and glands of Littré, as seen in man, were also present (fig. 2).

*The Mare.*—The stratified squamous epithelium at the external orifice changed to stratified columnar and then to transitional toward the bladder (fig. 3). Slight secretory activity was seen in the surface cells.

* Fisher Scientific Co., Pittsburgh, Pa.

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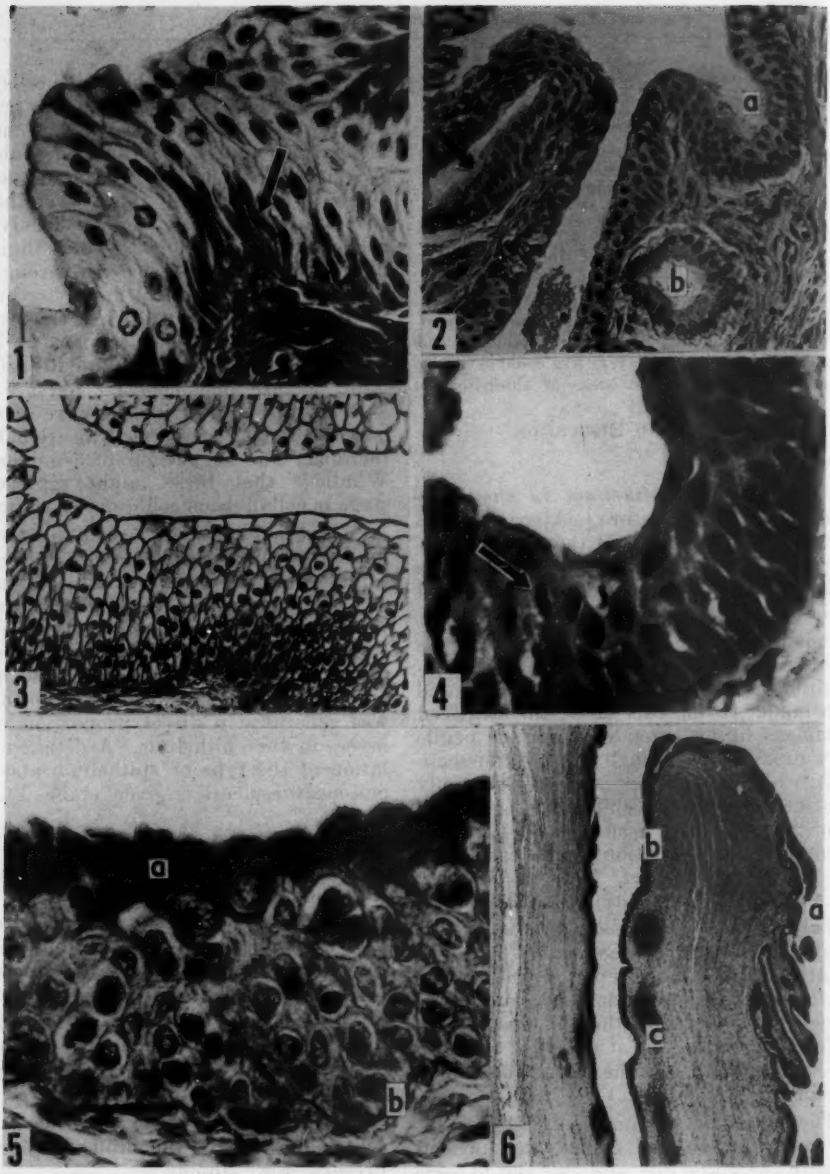


Fig. 1—Penile urethra of the stallion showing an admixture of stratified columnar and transitional epithelium; fibroblast-like cells (arrow) extend from the lamina propria into the epithelium. Azan aniline blue stain; x 606.

Fig. 2—Pelvic urethra of the stallion showing mixed transitional and stratified columnar epithelium (arrow) and (a) lacunae of Morgagni and (b) glands of Littré. H&E stain; x 240.

Fig. 3—Urethra of the mare near the neck of the bladder, showing typical transitional epithelium. H&E stain; x 162.

Fig. 4—Urethra of the bull at the sigmoid flexure, showing transitional epithelium with prickle cells in the middle layers. H&E stain; x 650.

Fig. 5—Suburethral diverticulum of the cow, showing transitional epithelium with (a) PAS-positive surface cells and (b) PAS-positive basement membrane. Periodic acid-Schiff stain; x 805.

Fig. 6—Suburethral diverticulum of the cow, showing (a) folded urethral mucosa, (b) less folded mucosa of the diverticulum, and (c) lymphoid nodules. H&E stain; x 11.

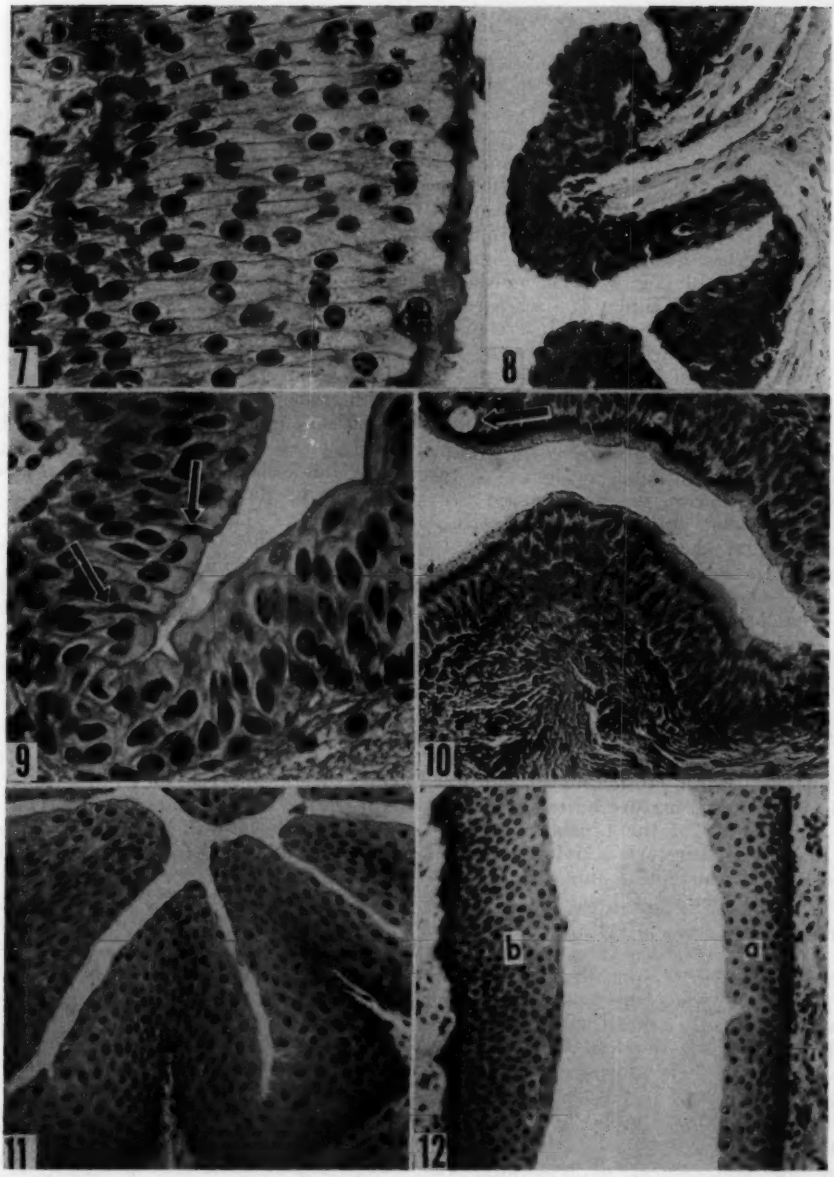


Fig. 7—Urethra of the cow near the bladder neck, showing transitional epithelium with elongated cells of the middle layer and a multinucleated surface cell. H&E stain; x 450.
Fig. 8—Processus urethrae of the buck, lined with transitional epithelium. H&E stain; x 212.
Fig. 9—Pelvic urethra of the buck showing transitional epithelium with "narrow cells" (arrows). H&E stain; x 605.
Fig. 10—Urethra of the doe showing stratified columnar epithelium and an intra-epithelial cyst (arrow). H&E stain; x 283.
Fig. 11—Processus urethrae of the ram lamb showing highly folded mucosa bearing transitional epithelium. H&E stain; x 144.
Fig. 12—Urethral lining in the glans penis of the ram lamb, showing (a) transitional epithelium on the side of the outer (ventral) thin wall of the urethra and (b) the thickened slightly papillated stratified squamous epithelium on the side of the urethral wall which rests on the bulk of the penis (dorsal). H&E stain; x 127.



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## CATTLE

*The Bull.*—Penile Urethra.—The entire penile urethra was lined by transitional epithelium resting on papillated connective tissue. Arey [1] noted a lack of intercellular bridges in transitional epithelium, but the prickle cells could be seen in the middle and basal layers, even beyond the sigmoid flexure in the bull (fig. 4). The epithelial pegs ("rete pegs," Bailey [2]) decreased in size and number and the intercellular bridges were rare in the bulbar part. The epithelium occasionally had marked indentations, the surface cells of which showed mucoid secretory activity.

Pelvic Urethra.—The cells of the middle layers of the transitional epithelium of the pelvic urethra were much elongated near the bladder and appeared to be stratified columnar, but the flat surface cells indicated its transitional nature. Similar epithelium occurred in females (fig. 7). The epithelial pegs were less prominent than in the penile part and rested on loose connective tissue with scattered lymphocytes and small lymphoid nodules. Trotter [21] also reported transitional epithelium in the pelvic and extrapelvic urethra, including the processus urethrae. He observed many lymphocytes in the epithelium of the processus urethrae of mature animals. Except for the opening of the prostatic ducts in the lumen, no secretory activity or glands were seen in the epithelium.

*The Cow.*—The stratified cuboidal or stratified columnar epithelium at the external urethral orifice was continued into the suburethral diverticulum. Proximally from the urethral orifice the epithelial lining changed to mostly transitional and resembled that already described in the bull (fig. 7). Some cells showed mucoid secretory activity, and intra-epithelial glands were also seen at the external urinary meatus.

In the suburethral diverticulum, the mucosa was less folded and papillated than the urethral side. The epithelium varied from stratified cuboidal or columnar to transitional and apparently rested on a basement membrane. The epithelial cell layers and heights varied from three to eight cells and 43 to 96 $\mu$, respectively. Lymphoid nodules were frequently seen below the epithelium (fig. 6). The surface cells showed secretory activity.

## THE GOAT

*The Buck.*—Penile Urethra.—The highly folded mucosa of the vermiform process bore transitional epithelium (fig. 8), and a similar epithelium extended throughout the penile urethra; toward the bulbous urethrae, patches of stratified columnar epithelium were present. Secretory activity seemed lacking in the entire penile urethra.

Pelvic Urethra.—The stratified columnar epithelium near the bulb was similar to the epithelium present in females (fig. 10). It changed to transitional proximally near the bladder, and was characterized by elongated cells like those in cattle. No evidence of secretory activity could be seen in the epithelial cells, but mucous glands opened into the urethral lumen.

*The Doe.*—The stratified squamous epithelium at the external orifice gradually changed to stratified columnar in the middle part (fig. 10) and finally became transitional near the bladder. The epithelium rested on a loose connective tissue rich in small blood vessels. No secretory activity was seen.

## THE SHEEP

*The Ram.*—Penile Urethra.—The processus urethrae were lined by transitional epithelium, as in the buck (fig. 11).

The urethral epithelium in the glans and the remainder of the penile urethra showed considerable differences in the young and adult animals studied. In the glans of the young animal, the urethral epithelium on the side toward the bulk of the glans was marked by prominent stratified squamous epithelial pegs. The epithelium resting on the opposite side toward the outer urethral wall was of a transitional variety (fig. 12). The remainder of the penile urethra was lined by stratified squamous epithelium in which intercellular bridges were maintained throughout.

In the adult animal, the entire penile urethra bore transitional epithelium (fig. 13). No evidence of secretory activity was found in any part of the penile urethra.

Pelvic Urethra.—The differences found in the penile urethra of young and adult animals occurred in the pelvic part (fig. 14, 15). In the young animal, the stratified squamous epithelium of the penile urethra extended proximally through the pars disseminata. The lining on the convexity of the crista urethralis was typical

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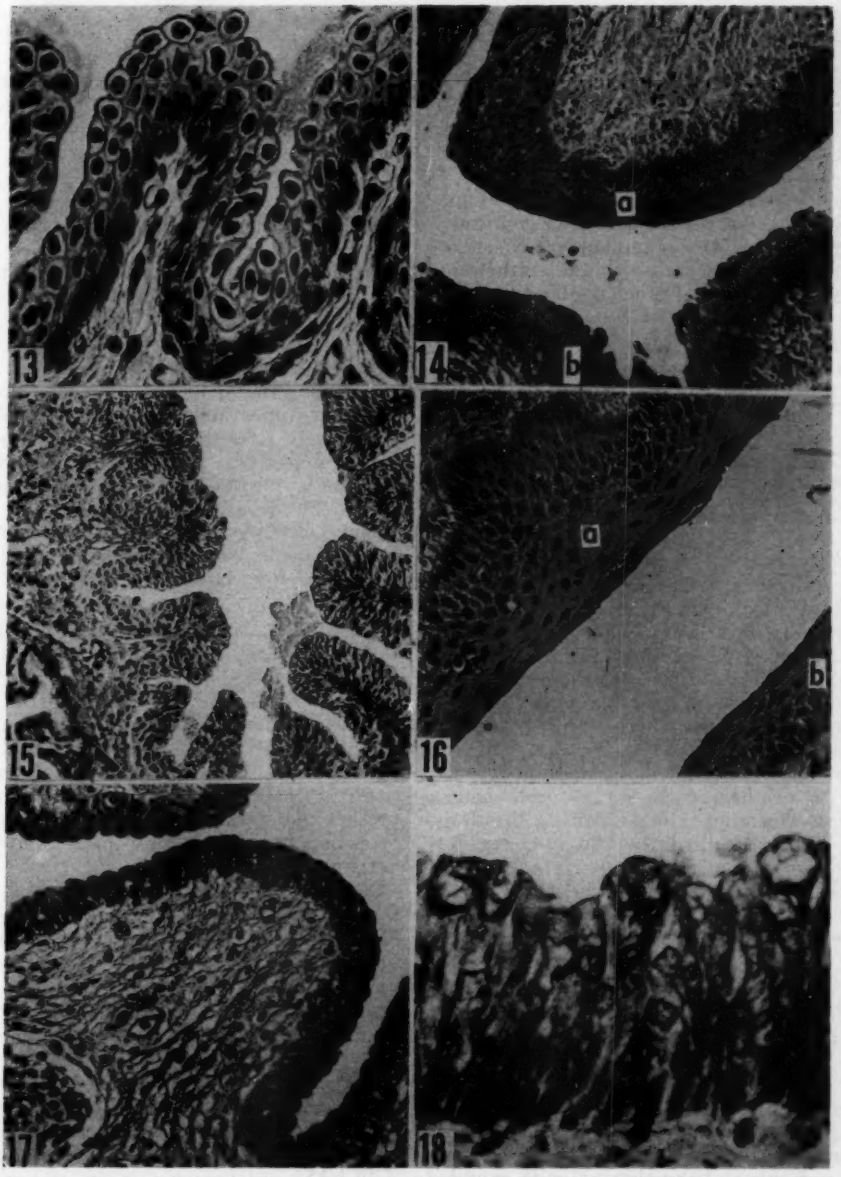


Fig. 13—Urethral lining in the glans penis of the ram showing the transitional epithelium. H&E stain; x 360.

Fig. 14—Pelvic urethra of the ram lamb showing (a) stratified squamous epithelium on the "crista" side and (b) transitional epithelium on the opposite side. H&E stain; x 120.

Fig. 15—Pelvic urethra of the ram showing the transitional epithelial lining and a portion of the prostate gland (arrow). H&E stain; x 247.

Fig. 16—Urethral lining in the tip of the penis of the boar showing (a) thick papillated stratified squamous epithelium toward the dorsal urethral wall and (b) a thinner and less papillated stratified squamous epithelium toward the ventral thin wall of the urethra. Notice the intercellular bridges in the thicker epithelium. H&E stain; x 218.

Fig. 17—Penile urethra of the boar, showing a transitional epithelial lining. Notice the beaded appearance of the surface cells, due to the large dense staining nuclei which fill the cells and make them bulge into the lumen. H&E stain; x 193.

Fig. 18—Urethra of the sow near the bladder showing secretory activity in the epithelium. Notice particularly the goblet cells. Toluidine blue stain; x 608.



XUM

stratified squamous with clearly visible intercellular bridges. The surface cells on the other side of the lumen were rounded and the epithelium assumed a transitional appearance.

In the adult animal, the transitional epithelium of the penile part was continued proximally as far as the bladder. No secretory activity was visible in the epithelium. Prostatic ducts and mucous glands opened into the lumen.

*The Ewe.*—The epithelium resembled that in the female goat, changing from stratified squamous at the orifice to stratified columnar to transitional. However, some cells showed mucoid secretory activity.

THE PIG

*The Boar.*—Penile Urethra.—Except for the stratified squamous epithelium at the spirally twisted terminal part of the external urethral orifice, the rest of the penile urethra was lined by transitional epithelium. The stratified squamous epithelium near the external urethral orifice was thicker and had more epithelial pegs on the side of the bulk of the penis (dorsal) than on the opposite side toward the thin outer urethral wall (fig. 16).

In the transitional epithelium of the penile urethra, each surface cell had a large, rounded, deep-staining nucleus, which almost filled the cell and made it bulge on the surface, presenting a beaded appearance in the lumen (fig. 17). Mucoid secretory activity was evident in the surface cells and occasional goblet cells, similar to those of the human cavernous urethra, were also present.

Pelvic Urethra.—Transitional epithelium, similar to that of the penile urethra, extended into the pelvic part. Near the bladder, the surface epithelial cells were much larger and stained more darkly; the middle layer cells appeared club-shaped with elongated nuclei; and the basal layer cells remained cubojdal or polygonal with rounded nuclei. No secretory activity was seen in the epithelium.

*The Sow.*—At the external urethral orifice, the surface cells of the stratified cuboidal lining became much enlarged and rounded proximally, and the epithelium appeared transitional in the remaining part, similar to that in the boar (fig. 17).

Near the bladder, the middle-layer cells became club-shaped and, in the enlarged surface cells, goblet cells with mucoid secretion were seen (fig. 18). Some cells in the recesses in the epithelium showed evidence of mucoid secretory activity.

THE DOG

*The Male Dog.*—Penile Urethra.—In the bone-free tip of the glans, the urethra was lined by an admixture of stratified columnar and transitional epithelium (fig. 19). In the rest of the penile urethra, the cell layers decreased and the epithelium assumed a stratified cuboidal, stratified columnar or, in some instances, a simple columnar appearance. The cells rested on a relatively thick layer of dense, homogeneous, noncellular connective tissue which had the appearance of a basement membrane. The free border of the surface columnar cells presented a thickened refractile appearance (fig. 21). Secretory activity was apparently lacking.

Pelvic Urethra.—The membranous part was lined by stratified cuboidal or columnar epithelium. The prostatic part had an admixture of stratified columnar and transitional epithelium. The lining on the side of the crista urethralis contained both transitional and stratified columnar epithelium, while the opposite side had only the transitional epithelium. Lymphoid nodules were present under the epithelium and lymphocytes migrating into the epithelium were not uncommon (fig. 20).

Near the bladder, the transitional epithelium had large surface cells (fig. 22). Mucoid secretory activity was seen in some upper layers of cells but intra-epithelial glands were lacking.

*The Bitch.*—The stratified squamous epithelium of the urethral tubercle immediately changed to stratified cuboidal or columnar which became transitional near the bladder. No secretory activity was seen in the epithelium.

THE CAT

*The Tomcat.*—Penile Urethra.—The stratified squamous epithelium at the external urethral orifice changed immediately to a stratified columnar type which was maintained in the rest of the penile urethra. Occasionally, mucous glands were seen opening into the urethral lumen (fig. 23).

Pelvic Urethra.—The membranous and prostatic parts had a mixed stratified columnar and transitional epithelial lining

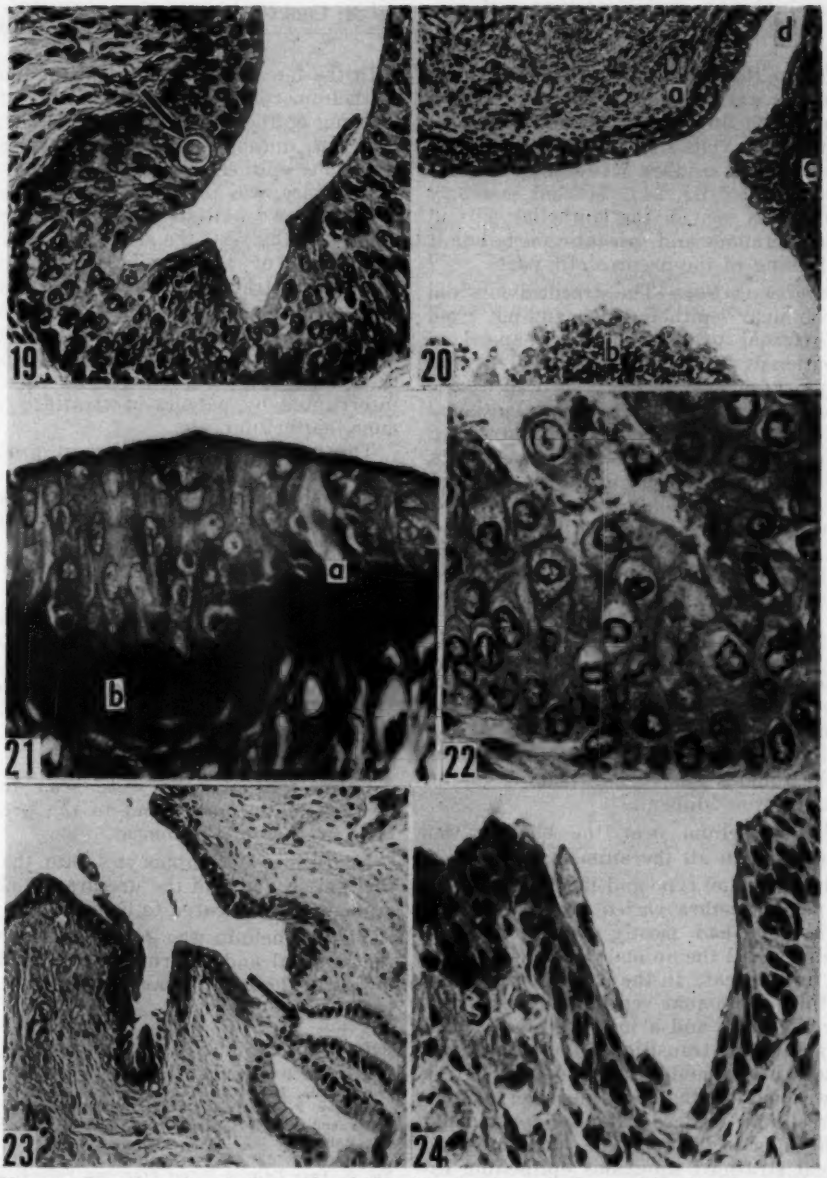


Fig. 19—Bone-free tip of the penis of the dog showing mixed transitional and stratified columnar epithelium and an intra-epithelial cyst (arrow). H&E stain; x 285.

Fig. 20—Prostatic urethra of the dog showing (a) mixed stratified columnar epithelium and transitional epithelium on the "crista" side, (b) transitional epithelium on the opposite side, (c) lymph nodule with lymphocytic migration in the epithelium, and (d) a prostatic duct. H&E stain; x 109.

Fig. 21—Penile urethra of the dog showing stratified columnar epithelium with a refractile-staining free border of the columnar cells, (a) basement membrane, and (b) homogeneous noncellular mass of connective tissue on which the epithelium rests. Weigert and Van Gieson stain; x 617.

Fig. 22—Pelvic urethra of the dog near the neck of the bladder showing transitional epithelium with large rounded surface cells. H&E stain; x 612.

Fig. 23—Penile urethra of the tomcat showing stratified columnar epithelium and mucous glands (arrow). H&E stain; x 194.

Fig. 24—Urethra of the queen showing a large surface cell. H&E stain; x 504.



XUM

similar to that in the dog. The pars preprostatica was lined entirely with a transitional epithelium which contained some large surface cells. The epithelium of this portion of the urethra was similar to that of the female (fig. 24). Mucoid secretory activity was seen in the epithelial cells in the membranous and prostatic parts but it was lacking in the preprostatic part.

*The Female Cat.*—The stratified cuboidal or columnar epithelium extending from the external urethral orifice changed to transitional near the bladder. The epithelium resembled that of the preprostatic part of the male but had fewer epithelial pegs and contained patches of stratified squamous epithelium. Large surface cells, like those in the preprostatic part of the tomcat, were commonly seen (fig. 24). No secretory activity was seen in the epithelium. Mucous glands similar to those in the penile urethra of the tomcat were also present at the external urethral orifice.

## SUMMARY AND CONCLUSIONS

The urethral epithelium from 41 animals of seven species was studied. Although the epithelium differed structurally in different species, at certain places the epithelium in all the animals was similar, making species differentiation difficult.

The epithelium near the bladder was transitional in all the animals.

The epithelial type and its extension into the penile urethra varied greatly, but the pelvic part had mostly transitional epithelium in all the animals except the buck, dog, and tomcat. In the latter species, only stratified columnar epithelium occurred near the bulb, and a mixture of stratified columnar and transitional epithelium was present in the middle part. In the bull, a transitional epithelium characterized by intercellular bridges extended proximally to the sigmoid flexure. The ram lambs had typical stratified squamous epithelium extending from the penile to the pelvic urethra which changed to transitional near the bladder. Their epithelium was the highest of all the male animals studied. Of the rest of the males, in both pelvic and extrapelvic parts, the bull had the highest epithelium, followed in decreasing order by stallion, dog, boar, buck, ram, and tomcat. The height was generally in direct proportion to cell layers in the epithelium.

In the females, the mare had the highest epithelium; other animals in order of decreasing epithelial heights were: ewe, cow, doe, sow, queen, and bitch. The stratified squamous epithelium, at the external urinary orifice, was present only in the mare, doe, and ewe and to some extent in the queen; in the rest, the stratified squamous epithelium of the vestibule immediately changed to stratified cuboidal or even transitional at the urethral orifice. There was marked resemblance in the epithelium of the ewe and doe, and bitch and queen. In the queen, the transitional epithelium was interrupted by patches of stratified squamous epithelium.

The part of the urethra most prone to expansion had a transitional lining irrespective of the type of epithelium proximal or distal to it, or on the surface opposite to it.

Both intra-epithelial cysts and "narrow cells" were frequently encountered.

Fibroblast-like cells were seen extending from the lamina propria into the epithelium of cattle and horses. Their exact nature and function could not be established. Mucoid secretory activity was frequently seen in the surface cells, and goblet cells were also present.

Large surface cells were seen in the urethra of the queen and in the preprostatic urethra of the tomcat.

Migration of lymphocytes into the epithelium occurred in the urethrae of all the animals and appeared to be normal.

The epithelium was generally highest at the internal and external orifices and was lowest in the middle part of the urethra in both sexes.

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# A Study of Discontinuity of the Fibula in the Horse

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BEGINNING ABOUT 1936, the author occasionally noticed a discontinuity of the fibula on radiographs of the knee joints of horses and diagnosed it as fracture. Eventually, it was found that such discontinuities appeared in healthy animals, and this created doubt as to their being fractures.

Radiographs taken after treatment was finished and lameness had disappeared showed that an interrupted course in the fibula still existed. The site of the defect was most often found in the area of the proximal epiphysial groove, thus giving rise to the assumption of a nonossified epiphysial cartilage.

There were, however, defects in more distal locations. Furthermore, some double or even multiple defects were observed, with irregularly dented and oblique outlines and varying widths of interspace between the "fragments," thus indicating that all discontinuities could not derive from a nonossified epiphysial cartilage. In addition, minute bone particles resembling fragments of bone appeared at the site of the discontinuity. Also, there were all stages of fusion of the separate parts of the fibula, rendering the problem of discontinuity of the fibula even more complex.

Überreiter [1] warned that radiography of horses often show epiphysial grooves in the region of the fibula which can be easily mistaken for fractures. Muller [5] described a discontinued fibula shown in a radiograph as a fracture. Reverchon and Brunot's description of joined "fractures" became known after the conclusion of the early course of this investigation, through the work of Delahanty.[2]

A paper preceding the work reported here described roentgenological examinations of the fibulae of 120 clinically sound horses of different ages; [3] it established the frequency and nature of the discontinuity of the equine fibula, which represents a rudimentary organ. These earlier examinations showed that the discontinuity of the fibula persisted in more than 50 per cent of the horses having faultless function of their limbs. About two thirds of such discontinuities were present in both fibulae. In about 10 per cent of the horses, defects appeared in two or more places. About two thirds of the defects corresponded to the proximal epiphysial groove, while the rest were more distal.

From such results, the conclusion was reached that discontinuity of the fibula is normal in this rudimentary bone. The article, published in Croatian (which is rarely spoken outside Yugoslavia), remained unnoticed. Shortly after its publication, several other papers appeared on fractures of the equine fibula accompanied by some degree of lameness; the etiology was not clearly defined and the clinical picture varied.[2, 4, 6] In these papers, fractures were described in more than 80 horses. All of these authors considered discontinuities of the fibulae in different cases of motor impairment as representing fractures. Therefore, it was decided to complement the roentgenological examinations we had already performed with anatomic and surgical investigations and have them published in English.[9]

Of 246 fibulae examined anatomically, complete synosteosis had occurred in only 30 per cent; partial joining of the bone was observed in 16 per cent. In the remaining 54 per cent, there was total disruption in the bone tissue either at the site where the capitulum of the bone passes into the corpus or within the corpus itself. Fibulae with several breaks in their course were separated by maceration into a number of parts. In the soft tissue lying between the bony parts of the fibulae, histological examination revealed various degrees of delay in the development of the epiphysial cartilage, necrosis of the cartilaginous tissue, irregular cavities, calcification of the decomposed cartilaginous mass, and degeneration of the cartilage cells with the appearance of collagenous fiber. The anatomical examination yielded results approximating those obtained with radiography. No callus was found on any of the specimens.

Surgical examination was performed in four artificial fractures of the fibula, one luxation with gross lesion of the corresponding part of the tibia, and one luxation with severance of the collateral ligament of the femorotibial joint. Directly following surgery in all these cases, there were some temporary disturbances in forward movement of the limb, manifested by slight dragging of the hoof along the ground. These were of short duration, however, and were ascribed to a dysfunction of the motor nerve, incurred with preoperative anesthesia or with trauma to one of its branches during surgery.

Attempts to fracture the bone by inflicting gross trauma over the uninjured skin met with failure. The surgical investigation demonstrated that neither fracture nor luxation of the fibula (if it occurs) can cause chronic lameness, as was assumed by some authors.

After the publication of this second article,[9] two further studies on the discontinuity of the fibula

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in the horse appeared in 1958. In one of them, Delahanty [2] suggested that the defects do not represent fractures, but rather disturbances in the development of the fibula. He also allowed for the possibility that muscle action and insertion of ligaments impede normal development of the bone, its ossification, and the joining of head and body of the fibula. Banks and Schulz [1] believed some discontinuities of the fibula to be caused by fractures, aside from congenital and etiological determinants. They reached this conclusion by observing the localization of the discontinuity and the appearance of necrotic and fibrocartilaginous tissue at such sites. Their opinion is at variance with the findings of our histological examination, according to which necrosis or fibrous tissue develops at the site of the defect in most cases of fibular discontinuity. Such a high percentage of fractured fibulae would hardly seem possible.

Our roentgenological examinations were resumed, the reason being that the first series of examinations comprised too few horses above 15 years of age and that 120 horses were too few to allow for establishing general conclusions. In the earlier examinations of 240 fibulae, it was observed that the fibula fused even after the fifth year; *i.e.*, at a time when osteogenesis of the other tubular bones had practically ended. The reduced number of examined horses and particularly the small number of old animals did not permit establishment of the duration of osteogenesis in this bone. We assumed that the equine fibula, because of its rudimentary character, is subject to certain biological specificities with, as their main feature, an incomplete and protracted osteogenesis. In order to solve the problem, earlier examinations were extended to include a larger number of horses.

## Experimental Procedure and Results

Roentgenological examinations were made of 400 horses (800 fibulae). Of the animals examined, 100 were 1 to 5 years old; 100 were 6 to 10 years old; 100 were 11 to 15 years old; and the remaining 100 were 16 to 20 years old.

In the horses that were 1 to 5 years old, the first synosteosis of the head and body of the fibula was observed in horses 2½ years old. Joined fibulae (one or both limbs) comprised 37.0 per cent of the total number of fibulae; 63.0 per cent were unjoined. In the unjoined fibulae, four fifths (79.6%) of the defects were bilateral and one fifth (20.4%) were unilateral. The continuity of the fibula was interrupted in one place in three fourths (75.5%) of the affected fibulae and in two or more places in one fourth (24.5%).

**Fig. 1—Oblique discontinuity of the fibula, corresponding in its site to the proximal epiphysial groove. Most defects (84.6%) occur in this location.**

In the horses 6 to 10 years old, 61.0 per cent of the fibulae were fused in one or both limbs and 39.0 per cent were unjoined. In unjoined fibulae, three fifths (59.2%) of the defects were bilateral and two fifths (40.8%) were unilateral. The continuity was disrupted in one place in four fifths (81.6%) of the affected fibulae and in two or more places in one fifth (18.4%).

In the horses 11 to 15 years old, 67.5 per cent of the fibulae were joined in one or both limbs and 32.5 per cent were not fused. As in the preceding group, three fifths (58.5%) of the defects were bilateral and two fifths (41.5%) were unilateral. The continuity was disrupted once in nine tenths (90.2%) of the affected fibulae and in two or more places in one tenth (9.8%).

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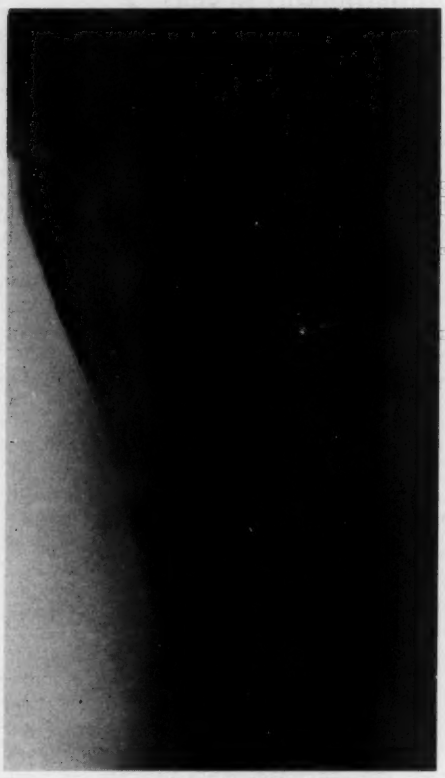

Fig. 2—Transverse discontinuity of the fibula. The proximal part of the unjoined fibula shows a bell-like deformity.

Fig. 3—Discontinuity of the fibula localized in the region of the diaphysis. Only 15.4 per cent of the defects are located there.

In horses 16 to 20 years old, 66.5 per cent of the fibulae were fused in one or both limbs, and 33.5 per cent were unjoined. As in the preceding two groups, three fifths (61.6%) of the defects were bilateral and two thirds (38.4%) were unilateral. Eight tenths (84.6%) of the fibulae had defects in one place only and two tenths (15.4%) had defects in two or more places.

By the end of the fifth year of age, 37 per cent of the fibulae had fused; from the sixth to the tenth year, another 24 per cent; and from the eleventh to the fifteenth year, 6 per cent. These data allow for the conclusion that osteogenesis of the equine fibula is prolonged and continues up to the fifteenth year of age. After that time, as statistics and graphs demonstrate, no further fusion of the separate bony parts of the fibula takes place. Two thirds of the fibulae join by the fifteenth year, whereas the remaining third are not joined by synosteosis at all.

Investigations of the discontinuity of the fibula on such a large number of animals have offered the opportunity of reaching more exact conclusions on the location of the defect. Most discontinuities (84.6%) were located in the region of the proximal epiphysial cartilage, while the others (15.4%) were in the region of the diaphysis. Accordingly, about five sixths of the defects corresponded in their localization to nonossified epiphysial cartilage.

That most discontinuities of the fibula represent sequelae of a disturbance in the ossification of the proximal epiphysial cartilage is also indicated by their occurrence in horses having a bilateral discontinuity. It has been noticed that in all horses with bilateral discontinuity, 90 per cent of the defects of either the left or right fibula

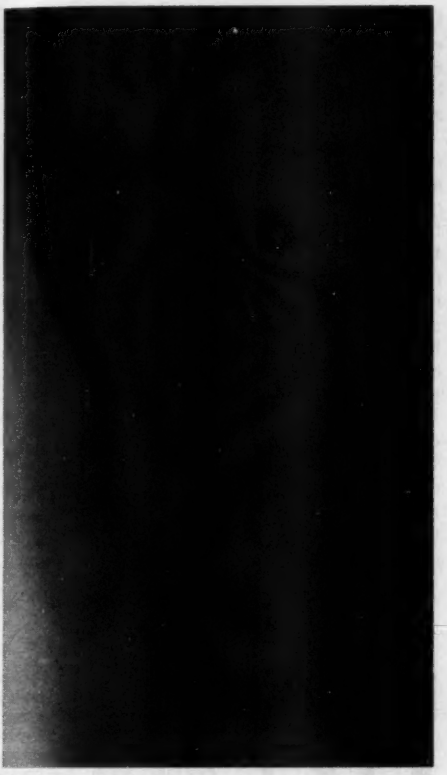

Fig. 4—A double discontinuity of the fibula in the region of the proximal epiphysial groove represents further proof of the abnormal ossification of the epiphysial cartilage.

Fig. 5—A double discontinuity of the fibula in the region of the proximal epiphysial cartilage and a simple defect in the region of the diaphysis indicate a complete disruption of the entire osteogenesis of the fibula.

were at the same height, corresponding to the epiphysial groove. The approximately equal distance between unjoined head and body of the bone in most of the horses (86%) is another indication of a nonossified epiphysial cartilage.

## Discussion

Roentgenological examinations demonstrate that ossification of the fibula may be protracted up to the fifteenth year of age. After that time, 33 per cent of the fibulae have a discontinuity either in the region of the proximal epiphysial cartilage or in the diaphysis itself. It should be emphasized that the osteogenesis of this bone is most intense up to 10 years of age; after that time, it drops rapidly to subside at the age of 15 years, which marks the period when osteogenesis ceases. Such protracted and incomplete osteogenesis, associated with a delayed and abnormal histological differentiation, forms the basis for believing that the discontinuities at the transition from fibular capitulum to corpus and in the corpus itself do not correspond morphologically to normal epiphysial synchondrosis.

With the exception of barely one fourth of all unjoined fibulae, the outlines of the defects in all the others are serrated, irregularly notched, convex, concave, or irregularly curved. In relation to the longitudinal axis of the fibula, most defects run obliquely, at an acute or obtuse angle. Because of the incomplete ossification of the epiphysial cartilage, the diastases between capitulum and corpus are often filled with more or less dense bone tissue, thus causing the outlines of the defects to be either barely discernible or to be surmised only

XUM

Fig. 6—Radiographs of isolated fibulae demonstrating clearly the serrated outline of the defects.

for their different structure and transparency. The clubbed thickening of the unjoined parts at the site of the defect, the interpolated particles of bone, and the partial synosteoses of varying degree (in addition to the described polymorphism) constitute the reason why the discontinuities may not be roentgenologically differentiated from genuine fractures. They are also the reason why, until quite recently, such discontinuities, which are normal for that bone, were considered as fractures by many authors.

Based on an analysis of the roentgenological examination of 800 fibulae in horses of all age groups, it is concluded that the discontinuity described can be explained only from the aspect of the theory of evolution. In the course of the phylogenetic development of the horse, the reduction of the number of digits has deprived the fibula of its basic function, thus eliminating it from the biological struggle and making it succumb to the process of rudimentation. At present, it is distinctly a rudimentary bone. Natural selection is known to be absent in rudimentary organs, so mutations occur without order. Consequently, there ensue disturbances in development and a much increased variability. The disturbances manifest themselves in retarded histological differentiation and growth of the organ, incomplete development, and absence of the final stage of ontogenesis. Such at-

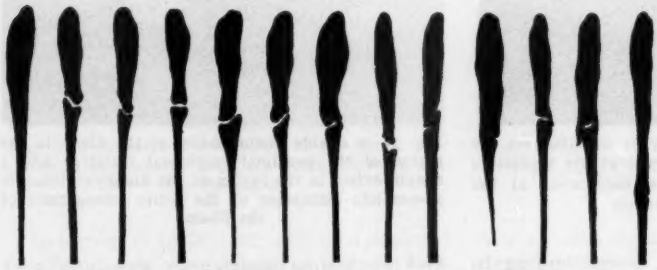

Fig. 7—Sketches of fibulae from radiographs showing different forms of discontinuity of fibulae with partial synostosis of varying degree. Such polymorphism occurs in more than three fourths of discontinued fibulae and represents the essential characteristic of rudimentary organs.

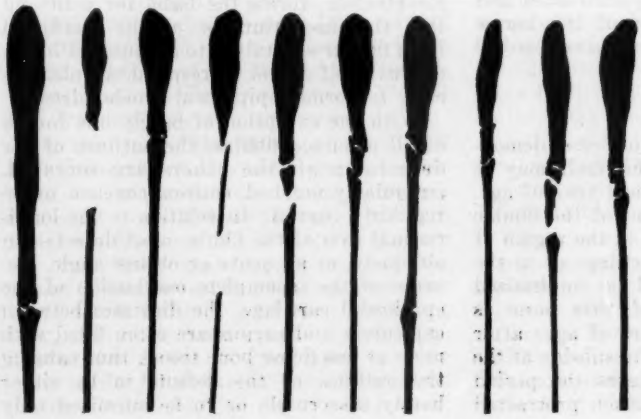

Fig. 8—Sketches of fibulae from radiographs showing varying numbers and sites of defects in the continuity of the fibula. Simple or multiple discontinuities of the fibula in one or both limbs represent a normal occurrence having no effect upon motor function.

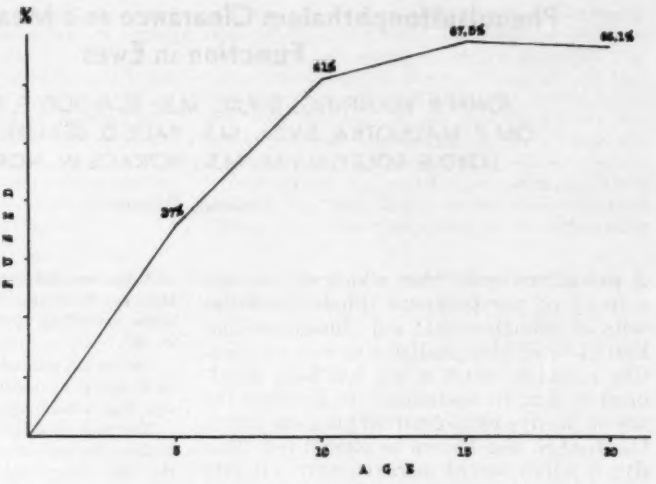


Fig. 9—Diagram showing relationship of osteogenesis of the fibula to age of the horse.

tributes of rudimentary organs, particularly the notably increased mutability, serve to explain all of the morphological deviations encountered in the equine fibula.

## Summary and Conclusions

On the basis of roentgenological examinations of the fibula in 400 horses, 1 to 20 years old, it was concluded that:

1) Discontinuities of the fibula in the region of the proximal epiphysial groove or diaphysis, whether unilateral, bilateral, single, or multiple, represent a normal phenomenon which does not affect the function of the limb in the least.

2) The course of ossification runs until the fifteenth year of age. By the fifth year, 37 per cent of the fibulae have fused; from the sixth to the tenth year, another 24 per cent, and from the eleventh to the fifteenth year, an additional 6 per cent. About 33 per cent of the fibulae remained unjoined.

3) Histological examination of the soft tissue lying between the bony parts of the fibula revealed retarded development of the epiphysial cartilage, degeneration and necrosis of the cartilaginous tissue, presence of collagenous fibers, and solid connective tissue.

4) The ontogenic development of the fibula in the horse is subject to certain phylogenetically acquired biological specificities with prolonged and incomplete osteogenesis as a main characteristic.

5) Incomplete osteogenesis and morphological deviations of the equine fibula are accounted for as occurring in a rudimentary organ in which the absence of natural selection results in the occurrence of mutations without order and causes increased variability, retarded growth, and absence of the final stage of ontogenesis.

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XUM

# Phenolsulfonphthalein Clearance as a Measure of Renal Function in Ewes

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A RENAL FUNCTION TEST which reflects the activity of the proximal tubule epithelial cells of animals would aid clinical evaluation in veterinary medicine as well as scientific research. Such a test has been developed in human medicine.[8] It involves the use of the dye phenolsulfonphthalein (PSP), $C_{19}H_{14}O_5S$, also known as phenol red. This dye is administered intravenously. Its rate of excretion in the urine indicates proximal tubule activity.

Mixner and Anderson [5-7] recently began a study with this dye in dairy cattle. Their work was confined to the blood plasma concentration of PSP as a measure of "fractional renal clearance." They defined fractional renal clearance as "the fractional part of the volume of distribution; as represented by blood plasma, which is cleared of dye per minute on an instantaneous basis."

This investigation was undertaken to determine whether the PSP excretion test could be adapted to the sheep. An investigation was made of plasma PSP concentrations as the sole criterion for estimating kidney function. A further investigation was made to evaluate the correlation of PSP in the urine with PSP loss from the plasma.

## MATERIAL AND METHODS

Six mature ewes (1, 2, 3, 4, 5, and 8) were used in this experiment. Each sheep was weighed. The rectal temperatures were recorded each day for three days. No febrile sheep was used. Blood samples were obtained from the jugular vein. The hemoglobin content was determined and total erythrocyte, total leukocyte, and differential leukocyte counts were made.[2] Urine from each animal was examined microscopically and for appearance and specific gravity.[3] Qualitative tests were made for sugar, pH, ketones, bile, and protein.[1]

A commercial PSP dye,* in a concentration of 6 mg. per milliliter was used. This solution was made according to the method described by Hawk *et al.*[4]

One to 1.5 gallons of water were administered to each sheep by stomach tube 12 hours before the dye was administered.

The first blood sample (20 ml.) was withdrawn before the animal was anesthetized and prior to the administration of the dye, using 20 mg. of sodium oxalate for each 10 ml. of blood.

Each sheep was anesthetized with sodium pentobarbital administered into the jugular vein. Light plane anesthesia was maintained by the addition of sodium pentobarbital when needed. A ventral incision was made on the midline in the lower abdomen over the bladder. This opening made possible the manual expression of urine from the bladder through a catheter inserted into the urethra of the sheep. The bladder was emptied by this method at the time the dye was administered.

The PSP was injected into the jugular vein at the rate of 1.5 mg. per pound of body weight. Immediately following the administration of the dye, a clean catheter was inserted through the urethra into the bladder. The free end of the catheter was placed in a bottle. Urine was expressed manually to empty the bladder simultaneously with the collection of two additional 20-ml. blood samples taken at slightly more than 30 and 60 minutes after the dye was administered.

Each experiment was terminated by euthanasia and necropsy of the animal. Gross pathological findings were recorded. Sections 2.5 by 2.5 cm. of liver and kidneys were placed in 10 per cent neutral formalin solution for 36 hours.

Liver and kidney tissues were processed by the paraffin method. Dehydration, clearing, and infiltration with paraffin were accomplished in an Autotechnicon.† Sectioning was done on a microtome ** set at 5 $\mu$ and placed on glass slides. The tissues were stained with hematoxylin and eosin.[9]

An additional female sheep (7) was used to determine how much PSP dye would be excreted in the urine in 24 hours. She was catheterized, the urine was removed, and PSP was administered intravenously at the rate of 1.5 mg. per pound of

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Based in part on a thesis submitted by the senior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the M.S. degree.

* Control E-2-A, Schaar & Co., Chicago, Ill.

† Autotechnicon, manufactured by the Technicon Company, Chauncey, N.Y.

** Spencer Model 820, manufactured by the American Optical Co., Instrument Division, Buffalo, N.Y.

body weight. She was placed in a metabolism cage with free access to feed and water and the urine was collected for a 24-hour period.

*Urinalysis for Phenolsulfonphthalein.*—Standards were prepared containing concentrations of zero to 0.0090 mg. of dye per ml. of standard solution. A few drops of 10 per cent NaOH was added to insure alkalinity. A spectrophotometer set at a wave length of 520 mμ was used to determine the optical density (o.d.) of the standards and urine samples. A standard curve was prepared.

The urine samples were diluted to 100 ml. with distilled water and stored for 24 hours. They were then filtered through coarse filter paper. A portion of the filtrate was diluted with distilled water to 100 ml. A few drops of 10 per cent NaOH was included in the dilution to develop the color of the PSP dye. The o.d. of each unknown was determined at 520 mμ. The concentration of PSP in the urine was determined and the percentage excreted was calculated.

*Blood Analysis for Phenolsulfonphthalein.*—The plasma samples from sheep 1 and 2 were analyzed for PSP according to the method of Mixner and Anderson.[7]

The following method represents a modification of Mixner and Anderson's procedure: Using the solution containing 6 mg. of PSP per millilter, standard solutions were prepared containing concentrations of zero to 0.0090 mg. of dye per milliliter; 4 ml. of plasma, 4 ml. of 10 per cent NaOH, and 2 ml. of water containing various concentrations of dye constituted the standard solutions. The o.d. of each standard solution was read from the spectrophotometer at a wave length of 560 mμ.[7] Distilled water was used to set the instrument at zero o.d. The o.d. of the dye-free plasma was subtracted from o.d. readings of the various concentrations and a standard curve was prepared.

Four ewes (3, 4, 5, and 8) were tested. The blood samples from each were centrifuged to obtain the plasma which was then stored in the refrigerator for 24 hours. The plasma was again centrifuged to eliminate the plasma clot which developed upon storing.

Four milliliters of plasma and 6 ml. of 0.5 *N* NaOH were placed in colorimeter tubes, mixed thoroughly, and let stand 10 minutes. Distilled water was used to set the spectrophotometer to zero o.d. The o.d. for the dye-free plasma sample was subtracted from the o.d. of the 30- and 60-minute samples to obtain the adjusted optical density (a.o.d.).

**TABLE 1—The Hemoglobin Content and the Erythrocyte, Leukocyte, and Differential Counts for 6 Sheep Used for Phenolsulfonphthalein Renal Function Studies**

| Item | Sheep No. 1 | 2 | 3 | 4 | 5 | 8 |
|---|---|---|---|---|---|---|
| BLOOD CONSTITUENTS | | | | | | |
| Hemoglobin* | 12.0 | 10.0 | 9.5 | 12.0 | 10.0 | 9.5 |
| Erythrocytes† | 6.81 | 6.51 | 6.47 | 9.77 | 6.23 | 7.29 |
| Leukocytes** | 8.4 | 7.7 | 12.3 | 8.7 | 11.1 | 10.0 |
| DIFFERENTIAL COUNT (%) | | | | | | |
| Eosinophils | 1 | .. | .. | .. | 5 | 2 |
| Stabs | 2 | 3 | 1 | 1 | .. | 2 |
| Neutrophils | 30 | 39 | 54 | 32 | 37 | 49 |
| Lymphocytes | 67 | 58 | 41 | 67 | 56 | 43 |
| Monocytes | .... | .... | 4 | .... | 2 | 4 |

* Gm./100 cc.; † millions/cmm.; ** thousand/cmm.

## RESULTS AND DISCUSSION

The 6 ewes showed no gross abnormality. All temperatures recorded were within the normal values for sheep (101.9–103.8).[3]

The hemoglobin content and the blood cell and differential counts of the 6 sheep used are shown (table 1). The variations

**TABLE 2—The Results of Qualitative Analyses of the Urine of 6 Sheep Used for Renal Function Studies**

| Item | Sheep No. 1 | 2 | 3 | 4 | 5 | 8 |
|---|---|---|---|---|---|---|
| Color | Yellow | Brown | Tea | Yellow | Straw | Yellow |
| Appearance | Clear | Flocculent | Clear | Cloudy | Clear | Clear |
| Specific gravity | 1.055 | 1.015 | 1.030 | 1.030 | 1.048 | 1.022 |
| pH | 8.0 | 7.2 | 8.0 | 8.0 | 7.6 | 7.2 |
| Microscopic | N | N | Few erythrocytes | Few pus cells | N | N |
| Qualitative tests | | | | | | |
| Protein | N | P | N | P | N | N |
| Sugar | N | N | N | N | N | N |
| Ketone | N | N | N | N | N | N |
| Bile | N | N | N | N | N | N |

N = negative, P = positive.

XUM

among the sheep are considered to be within the expected standard values.[2]

The qualitative analyses of urine samples from the sheep tested for renal function (table 2) show that the urine of sheep 2 and 4 contained protein. The urine of sheep 4 also contained a few neutrophilic polymorphonuclear leukocytes. The urine samples of sheep 1, 3, 5, and 8 were normal.[2]

**TABLE 3—Phenolsulfonphthalein (PSP) Plasma Concentrations and Fractional Renal Clearances**

| Sheep No. | Sampling time (min.) | Plasma PSP (mg./ml.) | $A_0$* | $-b_0$† | $T_{½}$** |
|---|---|---|---|---|---|
| | 33 | 0.011 | | | |
| 1 | 59 | 0.008 | 0.015 | 0.01290 | 53.7 |
| | 40 | 0.003 | | | |
| 2 | 69 | 0.001 | 0.013 | 0.03261 | 21.2 |
| | 37 | 0.003 | | | |
| 3 | 67 | 0.001 | 0.012 | 0.02750 | 25.2 |
| | 31 | 0.006 | | | |
| 4 | 62 | 0.003 | 0.012 | 0.02458 | 28.1 |
| | 32 | 0.009 | | | |
| 5 | 63 | 0.002 | 0.041 | 0.03758 | 18.4 |
| | 32 | 0.017 | | | |
| 8 | 62 | 0.003 | 0.110 | 0.04537 | 13.0 |

*$A_0$ = calculated concentration of PSP (mg./ml. of plasma) at zero time. †$-b_0$ = slope for PSP fractional clearance. ** Biological half-time or half-life of the disposal rate.

The gross necropsies and histopathological studies indicated that all 6 sheep were functionally normal. Limited granular cytoplasmic changes were present in the kidney tubular epithelium, except in sheep 5 and 8. It was concluded that these changes were too limited to have any appreciable effect on the kidney function.

Microscopic examination of the sections of kidney and liver of the 6 ewes revealed sheep 1 to have a liver essentially free from pathological changes. The kidney showed cytoplasmic granulation and an occasional necrotic cell, as evidenced by pyknosis. The liver of sheep 2 had a limited subacute portal hepatitis and the kidney had cytoplasmic granulation and some free albumin in the tubules. The livers of sheep 3 and 4 were apparently free of pathological changes and the kidney cells had cytoplasmic granulation. Sheep 5 and 8 were essentially free from pathological alterations in both liver and kidney.

**TABLE 4—Calculated Phenolsulfonphthalein (PSP) Excretion from Plasma Compared with Actual Excretion of PSP in the Urine**

| Sheep No. | Total inj. dose (mg.) | Sampling time (min.) | Dye excreted in urine (%) | Calculated % of dye lost from plasma |
|---|---|---|---|---|
| | | 33 | 55 | 28 |
| 1 | 128 | 59 | 68 | 48 |
| | | 40 | 60 | 77 |
| 2 | 159 | 69 | 69 | 92 |
| | | 37 | 54 | 75 |
| 3 | 180 | 67 | 61 | 92 |
| | | 31 | 46 | 50 |
| 4 | 275 | 62 | 56 | 75 |
| | | 32 | 45 | 78 |
| 5 | 221 | 63 | 55 | 95 |
| | | 32 | 73 | 82 |
| 8 | 180 | 62 | 90 | 97 |

In the 24-hour PSP excretion trial, sheep 7 excreted 92 per cent of the dye. This percentage is similar to those obtained in other animals and man.

The percentages of PSP excreted in the urine of the 6 sheep (table 4) shows that the average 30-minute excretion was 56 per cent ± 10 and the average 60-minute excretion was 67 per cent ± 13.

The excretion of the dye ranged from 55 to 90 per cent over approximately one hour. These rates of clearance are in fair agreement with the rate of clearance in other animals and man with functionally unimpaired kidneys.

In view of the healthy appearance of the animals together with apparently normal blood and urine findings, limited or negative gross and histopathological changes, and the results of the PSP excretion tests, these 6 sheep were considered to have unimpaired kidney function. Gross and histopathological studies also indicated unimpaired liver function. The literature failed to reveal attempts to establish functional normality in terms of these criteria. It is impossible in man and has been inadequately considered in animal kidney function experiments.

This investigation demonstrates that the PSP excretion test may be used to measure kidney function in the sheep. The excretion test also gives values which indicate normally functioning kidneys.

The results of blood plasma determinations of sheep 1 and 2 (table 3) are based on the methods of Mixner and Anderson,[7] who use an acid-alkali method of preparing the plasma for the o.d. reading. With this method a serious problem arises. A plasma sample is divided into 2-ml. portions and placed in two tubes. The addition of acid to one of these produces additional visible turbidity, while the addition of alkali to the other tube lessens the turbidity. If the acid tube is placed in the spectrophotometer

and set to zero o.d., the alkali tube would then have an o.d. of less than zero. Hence, this method should not be used with sheep plasma. This is not always the case, as evidenced by results obtained in sheep 1 and 2. The degree of plasma turbidity varies greatly from sheep to sheep. The acid method attempts to measure free hemoglobin in the plasma, which will interfere with the accurate determination of the amount of PSP in plasma. The addition of acid to the plasma containing free hemoglobin causes the formation of acid hematin. Proper handling of the blood during and after collection can also eliminate the occurrence of free hemoglobin in the plasma.

The method of Mixner and Anderson [7] was modified because of the frequent occurrence of turbidity in tubes containing acid and plasma. In the modification, distilled water was used to set the spectrophotometer to zero o.d. The substitution of distilled water for the tube containing plasma and acid eliminated the problem of turbidity. Only those plasma samples which apparently contained no hemoglobin were analyzed. The relation of o.d. to various dye concentrations in water was direct and rectilinear. However, varying concentration of dye in the standard solutions containing plasma and alkali resulted in a curve. Thus plasma standards must be used for the estimation of the dye concentration in plasma samples. The o.d. of dye-free plasma varies from sheep to sheep. This variation was taken into account in each set of analyses for plasma PSP.

The modified method was used to analyze plasma obtained from sheep 3, 4, 5, and 8 for PSP concentration (table 3).

The blood plasma data result from applying the methods of Mixner and Anderson [7] and modifications of these methods to sheep blood. They stated that PSP fractional clearance is easily measured and is considered to be a dynamic measure of renal function as indicated by plasma PSP concentration.

The three terms ($A_2$, $-b_2$ and $T\frac{1}{2}$) used by Mixner and Anderson [7] are used here to facilitate comparison. The $A_2$ is obtained by extrapolation of the disposal curve and represents the concentration of PSP (mg./ml. of plasma) at zero time. The mean $A_2$ for dairy cattle was 0.0146, with a range of 0.0034 to 0.0295. All except sheep 5 and 8 fell within this range and had values almost identical to the mean value for dairy cattle (table 3).

Mixner and Anderson [7] defined $-b_2$ as the fractional part of the volume of distribution, as represented by blood plasma, which is cleared of dye per minute on an instantaneous basis. They stated that this value is the measure of renal function. The $-b_2$ observed for cattle was 0.0224 to 0.0413, with a mean of 0.0298. For ewes, $-b_2$ was 0.0129 to 0.0453, with a mean of 0.0300.

The biological half time, $T\frac{1}{2}$, is obtained by dividing 0.693 (the natural logarithm of 2) by $-b_2$. The average $T\frac{1}{2}$ for cattle is 24.4 minutes. The average biological half time for these sheep was 26.6 minutes.

This paper presents data which are remarkably similar to data obtained by Mixner and Anderson,[7] although they were obtained from experiments with sheep rather than dairy cattle.

A comparison of calculated PSP excretion, based on plasma loss of the dye, with actual excretion of PSP in the urine (table 4) indicates much higher theoretical excretion values than were recorded in the urine test. The calculation of expected excretion based solely on blood plasma content of PSP is derived from the equation $A_t : A_2 :: x :$ total dose. The $A_t$ represents the concentration of PSP (mg./ml. of plasma) at the sampling time; $A_2$ represents the concentration of PSP (mg./ml. of plasma) at zero time by extrapolation of the disposal curves. The total dose is the amount of PSP administered; therefore, x represents the total dye remaining in the sheep at the specific time intervals.

The calculations for this comparison are based on the following assumptions:

1) $A_2$ is a valid extrapolation value.
2) Volume of distribution $= \frac{\text{mg. of dye injected}}{A_2}$
3) Volume of distribution at zero time equals volume of distribution at any time.
4) Surgical effects are negligible.
5) Urine excretion has little effect on volume of distribution.
6) Bile concentration of the dye does not appreciably affect the excretion of the dye.

Accepting these assumptions, the proportionality of the calculated dye loss from blood plasma to actual dye loss is reasonable. The variations between experiments are no greater than the urine value variations.

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In addition to the comparison in table 4, a statistical analysis of the slopes of urine excretion versus time on plasma loss of dye versus time gave a regression coeffcient of $b = 0.005 \pm 0.198$. The slopes were determined by using the rate of excretion difference and dividing by time. Statistically, the deviation involved is so large that there appears to be no correlation between urine excretion of PSP and decrease in plasma dye concentration.

## SUMMARY

Phenolsulfonphthalein (PSP) was administered intravenously to 6 mature ewes. Clinical observations, blood and urine studies, and gross and histopathological examination of the kidneys and liver indicated that these animals were functionally normal. Urine and blood collections were made simultaneously.

The standard PSP excretion test revealed that the average 30-minute urine excretion of PSP in ewes was 56 per cent and the average 60-minute excretion was 67 per cent. These average percentages are compatible with 40 and 60 per cent excretions in man at 30 and 60 minutes, respectively. The results of the PSP excretion tests confirmed the clinical and pathological results, which indicated that the sheep had unimpaired kidney function.

Two methods of plasma analysis were used. Plasma from sheep 1 and 2 were done by the method used for dairy cattle. Plasma from sheep 3, 4, 5, and 8 were analyzed by a modification of that method. The method used for cattle seems to be undependable because of the occurrence of turbidity in sheep plasma. The modified method overcame this variation.

The sheep plasma PSP data were compared with cattle PSP data and were similar. The $-b_2$ value, defined as the fractional part of the volume of distribution as represented by blood plasma, which is cleared of dye per minute on an instantaneous basis, is almost identical. The mean $-b_2$ value for dairy cattle was 0.0298. The mean $-b_2$ value for these sheep was 0.0300.

A comparison of calculated PSP excretion, which was based on the plasma loss of the dye, with actual excretion of PSP in the urine was presented. The data indicated that the plasma loss of dye was higher than the actual excretion. A statistical analysis of the slopes of urine excretions versus time on plasma loss of dye versus time gave a regression coefficient of $b = 0.005 \pm 0.198$.

Additional investigation is needed to establish more clearly the validity of PSP disappearance from plasma as a method of measuring kidney function in the sheep.

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# Ruminant Urolithiasis: I. Preliminary Observations in Experimental Ovine Calculosis

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INFORMATION is incomplete concerning the origins, sites of excretion, and chemical interaction of organic and inorganic metabolites isolated from ruminant uroliths. The constant occurrence of urinary calculi in ruminants in California feedlots has suggested that certain feeds and environmental conditions predispose ruminants to calculosis. Newsom [14] recognized that mineral imbalance was an important etiological factor in ruminant urolithiasis and later concluded [15] that phosphorus *per se* was not directly responsible. Udall *et al.*[17] observed significant increases in the urinary level of the protein-bound hexosamine when the concentrate-to-roughage ratio was elevated between 4:1 and 8:1 and when the calcium level was subsequently increased to three times the National Research Council requirement.

That mucoproteins are important in the formation of urinary stones has been shown in the extensive investigations of Boyce *et al.*[3-6] In these studies, increased levels of urinary mucoproteins were observed in human patients with calculous disease. Chemical analyses of the matrices of human uroliths revealed a composition similar to some urinary mucoproteins isolated by ultrafiltration from urine.[4] Boyce *et al.*[3] have suggested that "activated" urinary mucoproteins chelate calcium from solution to form relatively insoluble colloidal micelles which may serve as "organic templates" in calcigerous stone formation. Keeler and Swingle[9] have recently reported results concerning analyses of the organic matrices of siliceous bovine calculi. Of special interest was the presence of sialic acid and absence of deoxypentose in contrast to the findings of King and Boyce[10] in the matrices of calcareous stones from man.

Lindley *et al*,[12] in 1953, used a factorial arrangement of experimental feeding to assess the importance of various electrolytes in the production of calculi. They reported that the supplementation of 2.5 per cent $K_2HPO_4$ to a diet with a concentrate-to-roughage ratio of 4:1 produced calculi in wether lambs. The greatest incidence of calculosis resulted in sheep supplemented with both potassium and phosphorus, rather than each singly. A high incidence of ruminant urolithiasis in South Africa has been observed to be associated with the ingestion of the karroo bush *(Acacia horrida)*, which contains a high potassium concentration.[8]

The present investigation presents preliminary observations concerning various blood and urinary constituents of sheep given an experimental diet similar to that described by investigators from the State College of Washington.[7, 12]

## MATERIALS AND METHODS

For the production of experimental calculosis, wethers were fed a pelleted diet containing the following components: wheat straw (20.0%), dried beet pulp (30.0%), linseed meal (17.0%), oats (15.0%), wheat (15.0%), NaCl (0.5%), and $K_2HPO_4$ (2.5%). Analysis of the pellets revealed the presence of ingredients in the following percentages: moisture, 7.13; fiber, 13.5; ash, 8.16; protein, 11.94; fat, 2.0; nitrogen-free extract, 57.3; calcium, 0.18; magnesium. 0.37; phosphorus, 0.71; potassium, 1.82; and sodium, 0.43. This diet contained 40 per cent less calcium than the calculi-provoking diet described by Elam[7] in 1957. Acetazolamide (Diamox*) was administered to three groups of wethers to assess the effect of a diuretic on the experimental production of urolithiasis. Thirty-five yearling wethers were allotted to five groups. Animals in groups 1, 2, and 3, each composed of 5 wethers, were fed the experimental diet plus a capsule (given *per os* daily) of 100, 200, or 400 mg. of acetazolamide, respectively. The 16 wethers in group 4 were given the experimental diet alone and control group 5, composed of 4 wethers, was provided with alfalfa hay ad libitum.

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* The Diamox was supplied by the American Cyanamid Co., Pearl River, N.Y.



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Twenty-four-hour urine samples were collected from sheep placed in metabolism stalls. Collection bags were prepared from commercial fountain syringes (2-quart size) which were slit partially open to provide a funnel-type collector to cover the prepuce. Collection apparatus and glassware used in the analysis of urine electrolytes were rinsed repeatedly with triple-distilled water following a detergent wash.

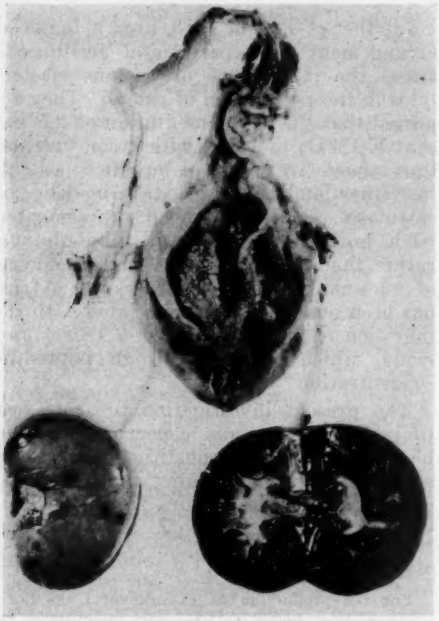

**Fig. 1—Excised urinary system exhibiting urethral occlusion and dilatation of renal pelvices. Notice calculous sediment in urinary bladder and urethra of wether fed the experimental ration. Rupture of the bladder was present (not shown).**

The measurements of serum or urine calcium, phosphorus, sodium, potassium, chloride, $CO_2$ combining power, and nonprotein-nitrogen were all performed by conventional procedures. Urine alkalinity was measured by a Beckman glass electrode pH meter.† The "microcalculi" in urine sediments and crushed calculous material were stained by the periodic acid-Schiff (PAS) technique [11] to identify possible polysaccharide-containing materials by the oxidation of their glycol groups to aldehydes, and by von Kossa's technique [11] to identify phosphatic accumulations.

Urinary mucoproteins for use in the preparation of standards for the estimation of urinary and serum mucoproteins were isolated from the urine of sheep given an alfalfa pelleted ration. A technique similar to that described recently by Anderson and Maclagen [2] for the isolation of human urinary mucoproteins was used. Following the addition of sodium benzoate to filtered acidified urine (pH 4.5), a white precipitate was collected in a Buchner funnel and subsequently washed free of benzoate with acetone. This precipitate was next dissolved in an aqueous solution of NaOH with a pH near 11.0, since the mucoprotein was insoluble at the lower pH of 9.0 as used in the isolation of human urinary mucoproteins. Following centrifugation (2,000 x g for 30 min.) of the alkaline protein solution, the supernatant fluid was dialyzed for 24 hours against repeated changes of distilled water using cellophane tubing* with constant rotation at 1 r.p.m. in a 1-liter cylinder. NaCl was added to a concentration of 0.2 M followed by the addition of five volumes of acetone to precipitate the mucoproteins. After three washings of the mucoprotein fraction with acetone, the centrifuged precipitate was dried in a vacuum oven overnight. The resulting hard, gummy pellet was dissolved in 6 to 10 ml. of 0.2 N NaOH and dialyzed in distilled water overnight. The mucoproteins were again reprecipitated with NaCl and acetone, centrifuged, washed repeatedly with acetone, dried under vacuum, and finally ground in a fine brown powder. In this manner, 130 mg. of mucoproteins were isolated and recrystallized from approximately 4 liters of ovine urine.

Urinary mucoproteins were analyzed for their sialic acid concentration by a method of Winzler,[10] using a standardized solution of pure n-acetyl sialic acid.

Routine determinations of urinary mucoproteins were performed by a method similar to that described by Anderson and Maclagen,[2] using 40 ml. of filtrate following initial precipitation and removal of any heat-coagulable protein with sulphosalicylic acid. Mucoproteins were estimated colorimetrically by the reaction of their sialic acid component with diphenylamine and expressed as milligrams of mucoprotein per 100 ml. of urine. Standard curves were prepared using 0.3 to 6.0 mg. of mucoprotein, which allowed for accurate estimation between a concentration range of 10 to 50 mg./100 ml. Three individually prepared mucoprotein isolates exhibited similar optical densities with the diphenylamine reagent and suggested that the sialic content of this urinary mucoprotein fraction remained reasonably constant on a diet of pelleted alfalfa hay. A pooled urinary mucoprotein sample from these 3 sheep was found to contain approximately 10 per cent sialic acid.[10]

Serum mucoproteins were estimated by a method similar to that of Lockey *et al.*[13] The serum heat-coagulable proteins were first precipitated with freshly prepared 0.2 M sulphosalicylic acid and the resultant mucoproteins in solution were precipitated in 5 per cent phosphotungstic acid in 2 N HCl. The precipitate was next dissolved in water which had been adjusted to a pH of 10 with

† Beckman Glass Electrode pH Meter, Model G, Beckman Instruments, Inc., South Pasadena, Calif.

* Cenco cellophane tubing, manufactured by Central Scientific Co., Chicago, Ill.

0.2 N NaOH in contrast to a pH of 9 with 0.1 N NaOH in the method of Lockey *et al.*[18] for human serum mucoprotein. Polysaccharide-protein conjugates, which were isolated with phosphotungstic acid from serum in sheep on the experimental diet, would occasionally require a pH of 11 or 12 to enter solution. Occasionally this heterogeneous fraction from sheep would not dissolve in highly alkaline solutions of NaOH with a pH greater than 12. Estimation of the mucoprotein concentration of the serum and urine by the diphenylamine test was not affected appreciably by changes in pH up to 12. The serum mucoprotein concentration was read from a standard curve of an aqueous solution of ovine urine mucoprotein isolated as described previously. It was necessary, however, to use three times the quantity of filtrate from the ovine serum samples as employed from human serums,[18] to obtain optical densities with the diphenylamine reagent in the accurate range of the spectrophotometer. The standard curve was prepared employing a concentration range between 10 and 300 mg. of mucoprotein per 100 ml.

## RESULTS

*Clinical Observations.*—Signs of urethral blockage and uremia first appeared 14 to 15 days following the commencement of feeding in 3 wethers in the groups given the "calculi-provoking" diet. One wether of group 3 (400-mg. acetazolamide supplement) and 2 wethers in group 4 (fed the experimental diet alone) first exhibited the classical uremic signs of stiffness, hyperpnea, stranguria, oliguria or anuria, a quivering tail, and extreme depression when rupture of the bladder occurred. On the fifteenth day, considerable gritty calculous material was observed on the preputial hairs of over 50 per cent of the wethers on the experimental diets, in contrast to none observed in the group of wethers on alfalfa hay.

Blood samples for blood chemical determinations were taken at this time from wethers on the concentrate diets. The 3 sheep with signs of urethral blockage had nonprotein-nitrogen (NPN) levels of 166.0, 180.0, and 190.0 mg./100 ml. blood. The other 32 sheep had blood NPN levels of 17.0 to 56.0 mg./100 ml. Total serum calcium ranged between 8.1 and 13.0 mg./100 ml. with no difference among the groups. Serum phosphorus levels in wethers of all groups ranged approximately between 3.0 and 7.0 mg./100 ml., with the exception of 3 acetazolamide-supplemented wethers which had levels of 9.0 mg./100 ml.

Serum alkaline phosphatase determinations on the five groups revealed no statistically significant differences in enzyme activity between any groups and values ranged between 0.5 and 10.3 Bodansky units. Allcroft and Folley [1] took blood samples from sheep repeatedly and reported that considerable variation in phosphatase activity exists in individual sheep as well as among different sheep under the same environmental conditions. They concluded that serum phosphatase activities were without merit in the diagnosis of clinical bone and mineral disorders in sheep. No significant differences or deviations from the normal were found in the serum $CO_2$ combining power, sodium, chloride, or potassium in the five groups of sheep.

Average daily water consumption during the second week of feeding of the experimental diet was 4.9 liters per sheep per day for the acetazolamide-supplemented animals and 4.03 liters per sheep per day for the wethers on the experimental diet alone. Feed consumption for the first 12 days prior to the outbreak of urolithiasis for the acetazolamide-supplemented animals averaged 3.7 lb. per sheep per day as compared to 3.4 lb. per day for the wethers not given the acetazolamide.

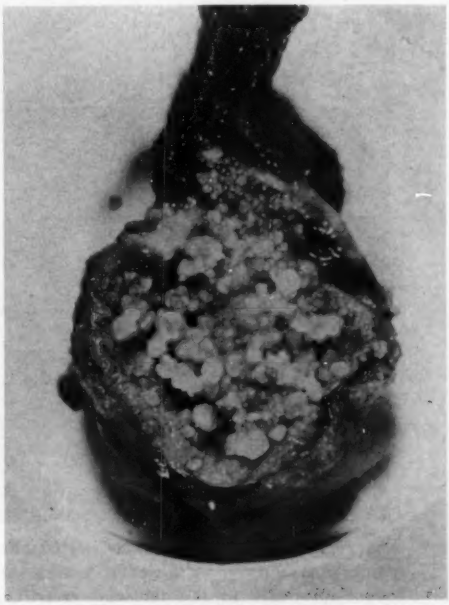

**Fig. 2—Urinary bladder of wether after having been fed the experimental diet for 18 days. Notice "crushed ice" consistency of calculous material filling the entire bladder.**

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During the physical examination of the processus urethrae (day 16) of all sheep given the concentrate diet, it was noticed that rupture of the processus had occurred from impacted calculi with resultant hemorrhage and inflamation of the Galea glandis in approximately 50 per cent of the wethers which appeared clinically normal. This rupture and subsequent release of urine may well have prevented the uremia syndrome as observed in other wethers, with occlusion occurring only at the processus urethrae. Necropsy of killed uremic wethers in all affected groups revealed either a rupture or dilation of the urinary bladder (fig. 1) and subsequent ascites from urethral occlusion at either the urethral process or sigmoid flexure. No renal or ureteral calculi were observed macroscopically. In 1 wether, the entire bladder was filled with a gritty calculous "sludge" of "crushed ice" consistency with only partial occlusion of the urethral process (fig. 2). During the 22 days on the experimental rations, many animals in all four groups given the experimental ration suffered rupture of the urethral process from the calculous material.

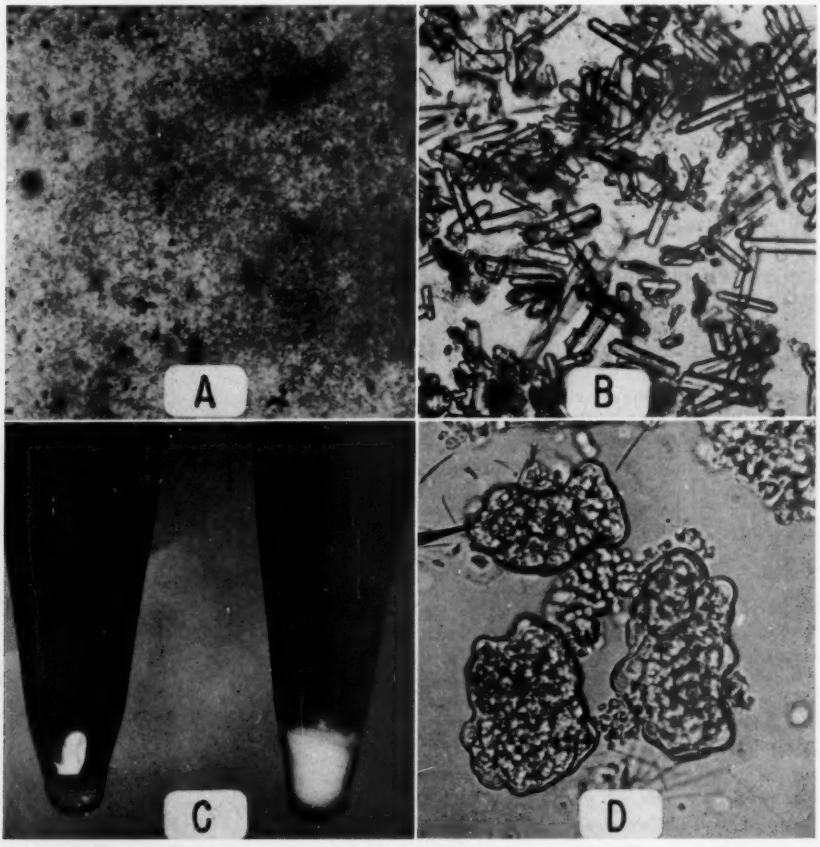


**Fig. 3—Urine sediment from wether fed alfalfa pellets (A); urine sediment exhibiting phosphate crystals from wether after having been fed the experimental diet for two days (B); macroscopic sediment (C) following centrifugation in 15 ml. centrifuge tube from the wether shown in figure 3A on alfalfa diet (left tube) and from the wether shown in figure 3B on experimental diet (right tube); and aggregates of "microcalculi" observed in wethers exhibiting partial urethral occlusion after 15 to 20 days on the experimental diet (D). x 400.**

Two wethers with urethral obstruction from each of groups 1 and 3, which were given acetazolamide supplementation, were killed. One of the 5 wethers in group 2, given 200 mg. of acetazolamide, was found to have uremia and subsequently killed for necropsy. In group 4, urethal occlusion was observed in 4 sheep at necropsy. No obvious difference in the occurence of urolithiasis was evident between the acetazolamide-supplemented wethers and those on the experimental diet alone. No abnormalities were observed in the urine of the control sheep in group 5 on the alfalfa ration.

When 9 wethers from groups 1, 2, 3, and 4 had exhibited uremia and been killed, the remaining sheep were moved to pasture grass on the twenty-third day of the trial and observed. No more cases of urolithiasis occurred and no calculous material was observed on the preputial hairs. The latter was constantly observed in wethers on alfalfa hay in control group 5.

*Urine Examination.*—Microscopic examination of urine sediments of wethers on the experimental diets and during the clinical onset of calculosis was of special interest. Wethers fed alfalfa hay or pellets displayed a homogeneous microscopic sedi-

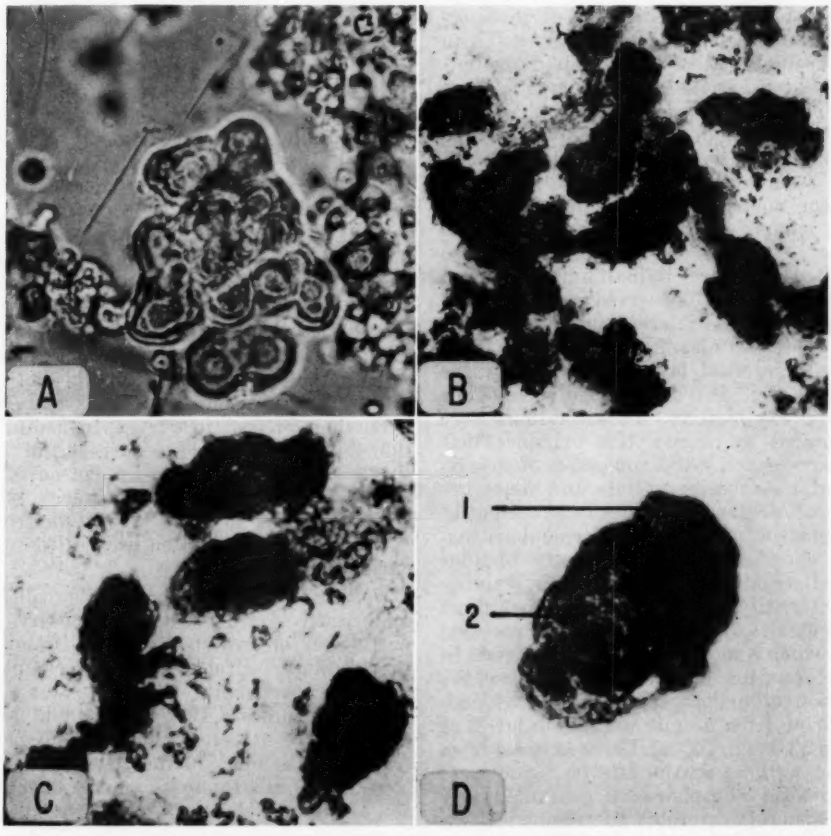


**Fig. 4—Urine sediments from wethers with experimental calculosis. (A) unstained aggregate of "microcalculi," x 900; (B) aggregates of "microcalculi," whose capsules and inner matrices stained positively with periodic acid-Schiff (PAS) reagents while their phosphatic composition counterstained intensely with the von Kossa's technique, x 200; (C) stained microcalculi, von Kossa—PAS, x 400; and (D) aggregate of "microcalculi" with darkly staining von Kossa-positive material (2) with PAS-positive envelope (1) and matrices, x 900.**

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ment in freshly voided urine which was free of phosphate crystals (fig. 3a). Urine pH was 8.2 to 8.5 in control sheep on the ration of alfalfa. Twenty-four hours after being given the concentrate diet, wethers excreted myriads of triple phosphate crystals in freshly collected urine (fig. 3b). An obvious increase in the urine sediment was macroscopically apparent following centrifugation in a 15-ml. conical centrifuge tube (fig. 3c). No change in urine pH was observed in these experimental sheep following introduction of the experimental diet, and this suggested that the limit of urinary phosphate solubility had been exceeded.

At the time of the appearance of clinical signs of partial and complete urethral occlusion in 5 wethers, aggregates of a few to many round bodies (3–7 $\mu$ in diameter) were observed in the urine sediment. These aggregates of microcalculi, held firmly together in an organic homogeneous envelope (fig. 4a), were seen in urine sediments collected from wethers with partial occlusion or from the urinary bladders of wethers with complete occlusion at the time of necropsy. Crushed calculous material also exhibited structures indistinguishable from the "microcalculi" observed in the urinary sediments. These microcalculi were found to be composed of von Kossa-positive crystals with internal layers of a PAS-positive material (fig. 4b). The organic envelope was also PAS positive. These findings were interpreted as presumptive evidence that the microcalculi were composed of a mixture of phosphatic crystals and mucoproteins enclosed as aggregates in envelopes of mucoprotein. Since crushed calculous material obtained from the urinary bladder yielded structures similar in their staining characteristics, it is quite likely that these microcalculi represent small structural units which coalesce to form large masses in stone formation. Of special interest was the elevation of urinary phosphorus in blood-free urine from uremic wethers to levels of 16.5 to 53.2 mg./100 ml. Urine samples from control wethers on the alfalfa ration were negative for phosphorus or contained only trace amounts. Aerobic bacteriological examination of the urinary bladders of sheep with urethral occlusion revealed the absence of bacteria in most cases; however, in advanced dilatation or rupture of the bladder, either micrococci, hemolytic streptococci or *Escherichia coli*, or both, were isolated. Urinary bladders of the 4 unaffected sheep on alfalfa and 4 sheep on concentrate diets free of urolithiasis were all negative for the presence of aerobic bacteria.

Spectrographic analyses* of calculous material obtained from 2 wethers, 1 given the experimental diet plus 100 mg. of acetazolamide daily and the other on the experimental diet alone, agreed only in part with previous reports.[7] Material from both wethers contained an inorganic composition principally of magnesium and potassium phosphate. The orders of magnitude were in a ratio of 1:3:15 for the elements of phosphorus, potassium, and magnesium. Calcium was present at a concentration one tenth that of phosphorus. Trace amounts of boron, silicon, iron, aluminum, lead, and copper were also observed. Calculous material from 1 steer in a feedlot where a number were naturally affected showed, spectrographically, an identical composition as found in the experimental wethers, with the exception of the presence of a trace of manganese and an increased copper content.

The x-ray diffraction analyses of the experimental calculous material indicated it to be quite amorphous and to contain only trace amounts of crystalline calcium phosphate. A previous report by Elam[7] described uroliths containing considerable calcium on a similar experimental diet containing two and one half times more calcium than present in our experimental ration. The more rapid occurrence of urolithiasis, as well as the lower concentration of calcium in the calculous material, could well be related to the lower calcium present in the pelleted ration used in the present investigation.

*Collection Trials.*—Urine was collected quantitatively from wethers to observe the pattern of electrolyte excretion following the feeding of alfalfa pellets, as compared to the experimental ration. These data (table 1) indicate that sodium and phosphorus were excreted in a greater concentration in the urine of the 2 wethers on the concentrate diet than observed in 3 wethers fed alfalfa pellets.

In two additional trials, in which each wether was changed from the alfalfa diet to the experimental diet, a marked increase in urinary sodium and phosphorus excretion was also observed in approximately

* The authors thank W. C. Theilen of the Public Health Spectrographic Laboratory, Berkeley, Calif.

24 hours. In two collection trials on alfalfa pellets, the carbonic anhydrase inhibitor (acetazolamide) at an oral dose of 80 and 1,000 mg. transiently increased urinary phosphorus excretion on the average of 20 and 100 times, respectively, 3 to 12 hours after its administration. No significant deviations occurred in the serum electrolytes.

Additional trials with quantitative urine collection were undertaken to compare both serum and urinary mucoprotein levels in wethers given alfalfa hay and the pelleted "calculi-provoking" diet. Ten yearling wethers fed alfalfa hay excreted in the urine an average of 218.0 ± 90.0 mg. of mucoprotein per day. Eight yearling wethers given the "calculi-provoking" diet excreted an average urinary mucoprotein of 341.0 ± 39.1 mg. per day. The average serum mucoprotein level of 10 yearling wethers on alfalfa hay was 35.2 ± 11.7 mg./100 ml., while 10 wethers given the experimental diet for three weeks presented an average serum level of 71.3 ± 16.9 mg./100 ml. Upon return to alfalfa hay for three weeks, the average serum mucoprotein level in these wethers dropped to 49.6 ± 17.6 mg./100 ml. Differences in the serum concentration and daily urine mucoprotein excretion between the wethers fed the two diets were highly significant ($p = <0.01$). Serum mucoprotein was expressed in terms of urinary mucoprotein standards for comparative purposes. That the mucoprotein of the serum and urine fractions both contain sialic acid is obvious from their reaction with diphenylamine; however, it has not been established as yet if their sialic acid concentration is similar or if identical polysaccharide-protein conjugates are common to both. These serum mucoprotein values should therefore be considered estimates at best. During each urine collection trial for the isolation of mucoproteins from wethers fed the experimental diet, animals became uremic from urethral occlusion two to three weeks after the commencement of feeding.

## DISCUSSION

The presence of increased urinary mucoproteins in wethers given "calculi-provoking" diets suggests a probable relationship between diet and calculi, as well as mucoproteins and the incidence of calculi. Of special interest was the elevation in the serum mucoprotein fraction associated with

**TABLE 1—Urinary Electrolyte Excretion Per Day in Wethers Fed Alfalfa Pellets or Experimental Diet Pellets Ad Libitum**

| Trial No. | Weight (kg.) | Diet | Days on trial | $HCO_3^-$ | | Cl | | Na | | K | | $HPO_4^=$ | | Av. urine vol./day (ml.) | Av. water intake/day (ml.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $\bar{X}$* | R† | $\bar{X}$ | R(mEq./day) | $\bar{X}$ | R | $\bar{X}$ | R | $\bar{X}$ | R | | |
| 1 | 35 | Alfalfa pellets | 5 | 278 | (161–305) | 324 | (267–381) | 23.2 | (8.88–43.1) | 581 | (370–816) | 1.77 | (1.24–3.1) | 977 | 3,784 |
| 2 | 40 | Alfalfa pellets | 5 | 257 | (130–383) | 317 | (242–381) | 21.2 | (0.96–52.8) | 317 | (180–458) | 2.48 | (0.0–2.84) | 922 | 2,940 |
| 3 | 35 | Alfalfa pellets | 5 | 258 | (231–272) | 275 | (193–350) | 1.85 | (1.3–2.5) | 868 | (763–1,029) | 0.0 | 0.0 | 1,072 | 5,070 |
| 4 | 35 | Concentrate pellets | 5 | 266 | (235–346) | 238 | (158–348) | 76.9 | (1.2–151.0) | 614 | (456–812) | 31.5 | (10.0–92.8) | 938 | 5,240 |
| 5 | 35 | Concentrate pellets | 5 | 302 | (262–351) | 255 | (168–349) | 97.0 | (70.0–153.0) | 565 | (466–657) | 37.2 | (13.0–93.0) | 925 | 5,225 |

* $\bar{X}$ = mean per day; † R = range during five-day period.

XUM

increased levels of urinary mucoproteins on the experimental diet. This finding suggests the additional possibility of a prerenal origin of certain polysaccharide-protein conjugates present in urine, as originally postulated in man by Anderson and Maclagen.[2] Variations in serum glycoproteins in inflammatory diseases, neoplasia, and following parathyroid extract administration have been recently reviewed.[16]

In the present study, as well as in other investigations,[17] mucoproteins are expressed in terms of one constiuent within the mucoprotein fraction. Since the mucoproteins are not determined gravimetrically, increases in optical density of this one component are interpreted as representative for the entire heterogeneous mucoprotein fraction. Increases in the percentage of sialic acid or hexosamine in specific homogeneous conjugates within this heterogeneous fraction could lead to error if interpreted as an increase in the entire complex. It is quite possible that the increase in urinary mucoproteins on the experimental diet in the present investigation may represent additional new chemical entities from the urinary system or from a prerenal origin. It was of special interest that the serum mucoprotein fraction following ingestion of the concentrate diet was uniformly more insoluble than on a ration of alfalfa hay. Present investigations in our laboratory are primarily concerned with the chemical identity of these mucoproteins isolated by ultrafiltration from the serum and urine of wethers on various rations. An understanding of the exact relationships between specific serum and urinary mucoproteins and calculous disease in ruminants demands the isolation, identification, and understanding of the physiological role of individual mucoproteins, found either active in the production of calculosis or within the urolith *per se*.

## SUMMARY

A pelleted ration with a high ratio of concentrate to roughage (4:1) and containing 2.5 per cent $K_2HPO_4$ consistently produced a high incidence of urolithiasis in yearling wethers. Occlusion at the sigmoid flexure or urethral process with subsequent rupture of the bladder were common findings.

Oral supplementation with acetazolamide did not decrease the incidence of urolithiasis. Urine from wethers with experimental urolithiasis exhibited aggregates of phosphatic "microcalculi" bound in organic envelopes which stained positively by the periodic acid-Schiff technique.

Sheep fed the "calculi-provoking" diet excreted more sodium, phosphorus, and mucoproteins per day in their urine than was observed when given alfalfa hay. Serum mucoprotein levels were also increased in wethers fed the experimental ration.

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# Experimental Infection of Swine with *Brucella Neotomae*

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In 1957, Stoenner and Lackman [4,5] isolated a member of the genus Brucella from the desert wood rat, *Neotoma lepida*. While this organism resembled *Brucella suis* in many respects, certain differences were noted which they considered sufficient to warrant naming it as a new species, *Brucella neotomae*.

According to these authors, on initial isolation the organism did not require an increased $CO_2$ tension, produced $H_2S$ and exhibited high urease activity similar to *Br. suis* and, in reciprocal agglutinin-absorption tests, fell within the *Brucella abortus*-*Br. suis* group. However, it failed to grow on mediums containing either thionin or basic fuchsin in the usual concentrations used for typing Brucella. They further considered it to differ from *Br. suis* in producing a lower pH in certain carbohydrate mediums, and in comparative infectivity for mice and guinea pigs.

Cameron and Meyer [3] compared the oxidative metabolism of seven strains of *Br. neotomae* to that of representatives of the three usually recognized species of Brucella. They found that these strains showed qualitative and quantitative differences from *Br. suis* in the rates of oxygen uptake on certain amino acid substrates.

Although it is debatable whether these differences are sufficient to warrant giving species rank to this organism, its characteristics differ in some details from those usually attributed to *Br. suis*.

With the continued reduction in incidence of bovine brucellosis under the brucellosis eradication program and the increased effort anticipated in the control of swine brucellosis, the discovery of further wild animal reservoirs or of aberrant strains of Brucella assumes increasing importance. A case in point is the role of infected hares in the epidemiology of swine brucellosis in Denmark.[2]

In view of the close resemblance of *Br. neotomae* to *Br. suis*, it seemed indicated to determine the pathogenicity of *Br. neotomae* for swine. Accordingly, the current study was undertaken.

## Materials and Methods

*Culture.*—The culture of *Br. neotomae* used for inoculation of swine bore the strain designation 7E-164.* Upon receipt in our laboratory (Beal and McCullough), it was examined by the oblique lighting technique and was found to be a pure colony type and possessed characteristics which we associate with the SI (smooth-intermediate) phases of Brucella. It did not require an increased $CO_2$ tension for growth, and produced $H_2S$ and exhibited high urease activity similar to *Br. suis*. Serologically, it was indistinguishable from S (smooth) *Br. abortus* and *Br. suis*. Employing trypticase soy agar † as the basic medium, the lots of certified dyes (thionin and basic fuchsin) distributed by the World Health Organization, and the methods recommended by the Joint FAO/WHO Expert Committee on Brucellosis,[1] the culture yielded dye reactions appropriate for *Br. suis*. In this regard, however, it had a greater sensitivity to thionin than is usually encountered in strains of *Br. suis*. It did not grow on mediums containing thionin at a dilution of 1:100,000, but grew well on mediums containing this dye at a dilution of 1:125,000. It did not grow on mediums containing basic fuchsin in this concentration or even when the dye content was reduced to 1:250,000.

*Swine.*—Eight pigs (5 gilts and 3 barrows), 40 to 60 pounds in weight and negative to the agglutination test for brucellosis, were housed on grounds at Purdue University. They were observed over a two-week period before use in this study. Samples of blood drawn immediately prior to their exposure to *Br. neotomae* were culturally sterile and were again negative to the agglutination test for brucellosis.

*Preparation of Inoculum.*—Following single colony selection, cells grown for 24 hours at 37 C. on trypticase soy agar slants were suspended in 0.85 per cent NaCl. Using a turbidity standard previously prepared on the basis of plate counts, the density of the suspension was adjusted electrophotometrically to provide the desired number of organisms in a final volume of 5 cc.

*Experimental Procedure.*—Four pigs (3 gilts and 1 barrow) were inoculated intravenously (an-

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† Baltimore Biological Laboratories, Baltimore, Md.

terior vena cava) with $1 \times 10^9$ *Br. neotomae*. An additional 4 pigs (2 gilts and 2 barrows) were given $50 \times 10^9$ *Br. neotomae* by the same route.

Blood samples were taken from the pigs at weekly intervals after exposure and their serums tested for Brucella agglutinins using the Bureau of Animal Industry standard plate antigen; the dilutions tested were 1:25, 1:50, 1:100, 1:200, and 1:400. At the same time, 5 cc. of blood was obtained under aseptic conditions and cultured for Brucella, using tryptose broth as the culture medium. Subculture to plates of tryptose agar was made at five- to seven-day intervals. The plates were incubated at 37 C. in normal atmosphere for one week before they were discarded as negative. Blood culture bottles were incubated under similar conditions for a total of two weeks. As isolations were made, they were shipped by airmail to the National Institutes of Health for identification and study.

Beginning with the ninth week postexposure, the animals were killed and the following tissues cultured for Brucella: lymph nodes (popliteal, prefemoral, superficial inguinal, internal iliac, gastrohepatic, mesenteric, mandibular, and cervical), kidneys, spleen, liver, heart, lungs, uterus, and ovaries. Culturing was done by smearing the freshly cut surfaces of the tissues on individual plates of tryptose agar. The plates were then incubated at 37 C. in a normal atmosphere for one week.

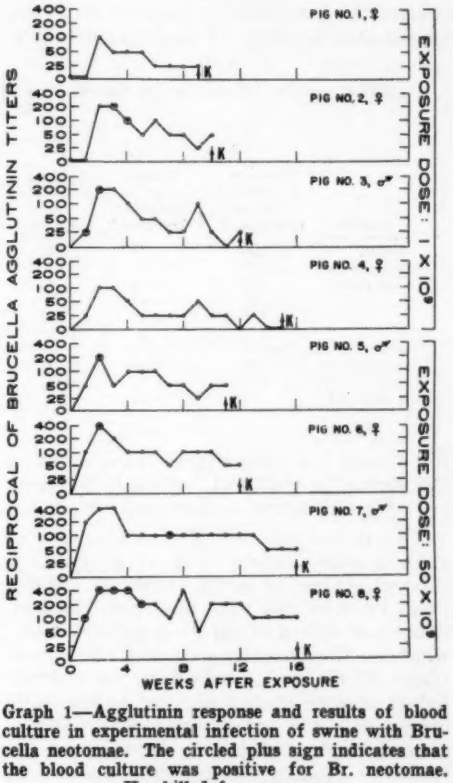
*Bacteriological Studies.*—All isolates recovered during the course of the experiment were subjected to bacteriological examination. Among the characteristics studied were: colony morphology, antigenic nature, sensitivity to thionin and basic fuchsin, hydrogen sulfide production, and urease activity.

For dye differentiation, thionin and basic fuchsin were incorporated in trypticase soy agar at final concentrations of 1:100,000, 1:125,000, 1:150,000, 1:200,000, and 1:250,000. Tests for $H_2S$ production were performed on slants of both liver infusion and trypticase soy agar. Urease activity was determined by the method of Bauer.[1]

The isolate representing the longest period of residency in swine (7 weeks) was tested for its infectivity for guinea pigs. For this purpose male guinea pigs, NIH strain, 300 to 350 Gm. in weight, were inoculated in groups of 10 with graded dilutions of standardized suspensions of the isolate. Suspensions were prepared from cells representing the second successive 24-hour growth from a single colony. The size of the inoculum was approximated turbidimetrically and the number of viable Brucella was determined by surface plating to trypticase soy agar. The inoculums, in a volume of 0.5 cc., were given intraperitoneally. After 30 days, blood samples were taken for agglutinins, the guinea pigs were killed, and the following tissues cultured for Brucella: spleen, liver, inguinal and axillary lymph nodes, and testes. The criterion of infection was recovery of *Brucella* at necropsy.

## RESULTS AND DISCUSSION

*Infectivity for Swine.*—The agglutinin response and blood culture results are shown (graph 1). All of the animals had developed Brucella agglutinins by the second week postexposure. Titers of 1:100 or higher were reached in all animals during the period of observation, and a demonstrable titer was maintained in all but 2 of




**Graph 1—Agglutinin response and results of blood culture in experimental infection of swine with Brucella neotomae. The circled plus sign indicates that the blood culture was positive for Br. neotomae. K = killed for necropsy.**

the pigs until termination of the experiment. Agglutinins occurred earlier, reached a higher level, and persisted longer in animals given the larger inoculum.

As determined by isolation of *Br. neotomae* from the blood stream two weeks or longer after exposure, infection was established in 6 of the 8 pigs. Probably the heaviest infection occurred in gilt 8, whose blood stream yielded positive cultures of *Br. neotomae* for five successive weeks.

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*Brucella neotomae* was isolated from the blood of 1 animal (barrow 7) seven weeks after exposure.

Upon necropsy, at the time indicated (graph 1), there was no gross evidence of infection and Brucella was not recovered from any of the animals.

*Bacteriological Studies.*—Twelve isolations of *Br. neotomae* were made from blood cultures of the experimentally infected swine. They were treated as individual cultures and studied in parallel with the parent strain. None of these cultures differed from the parent strain used to infect the swine, in cultural, colonial, biochemical, dye sensitivity, or antigenic character.

The infectivity of *Br. neotomae* for guinea pigs, before and after passage through swine, is given (table 1). While gross local lesions were observed, the incidence and extent of such lesions were somewhat less than usually seen with the smooth phase of *Br. suis*. Infections were established in some guinea pigs by as few as 25 organisms, and generalized infection occurred as attested by the recovery of *Br. neotomae* from a multiplicity of sites. In no instance was there failure to recover Brucella from a guinea pig showing agglutinins; the organism was recovered from 6 guinea pigs which failed to develop agglutinins. Residence in swine did not enhance the infectivity of *Br. neotomae* for guinea pigs.

It is apparent that *Br. neotomae* is infectious for swine by the intravenous route. The infections produced in this experiment appeared to be self-limiting. While the number of animals used was too small to warrant generalizations, infections seemed moderate in degree in comparison to those usually obtained with *Br. suis*. According to our criteria, the culture of *Br. neotomae* consisted of an SI phase of the organism. This culture was used in the experiment since we were unable to obtain the organism in an S phase (comparable to the S phase of other Brucella) by colonial selection, and such was not available from the authors who originally described the organism. This may explain the failure to produced prolonged infection with the exposure doses used.

**TABLE 1—The Infectivity for Guinea Pigs of Brucella Neotomae, Before and After Residence in Swine**

| Culture | Exposure dose (No. of organisms) | No. of guinea pigs infected/No. inoculated | Culture and agglutination results from guinea pigs at necropsy | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Tissues positive for *Br. neotomae* | | | | Brucella agglutinins | | |
| | | | Spleen | Lymph nodes | Liver | Testes | No. positive | Range of titer Max. | Range of titer Min. |
| Before exposure to swine | 25,000 | 8/10 | 7 | 7 | 5 | 3 | 6 | 1:640 | Neg. |
| | 2,500 | 8/10 | 7 | 8 | 6 | 2 | 8 | 1:10,240 | 1:20 |
| | 250 | 6/10 | 6 | 6 | 6 | 1 | 6 | 1:640 | 1:20 |
| | 25 | 3/10 | 3 | 2 | 1 | 0 | 2 | 1:640 | Neg. |
| | 2 | 0/10 | 0 | 0 | 0 | 0 | 0 | ..... | ..... |
| After 7 weeks residence in tissues of swine | 20,000 | 9/10 | 9 | 9 | 8 | 4 | 9 | 1:1,280 | 1:20 |
| | 2,000 | 4/10 | 4 | 2 | 2 | 1 | 3 | 1:2,560 | Neg. |
| | 200 | 3/10 | 1 | 1 | 0 | 2 | 1 | 1:80 | Neg. |
| | 20 | 0/10 | 0 | 0 | 0 | 0 | 0 | ..... | ..... |

The characteristics of *Br. neotomae* which distinguish it from classic *Br. suis* strains are sufficiently stable so that no change was detected after residence in swine.

## SUMMARY

Pigs were experimentally exposed to *Brucella neotomae* by the intravenous route. Infection was established in all, and bacteremia was demonstrated in 2 of 4 pigs inoculated with 1 billion organisms and in all of 4 pigs given an inoculum of 50 billion. Bacteremia in 1 animal existed for five successive weeks and in another *Br. neotomae* was isolated from blood culture seven weeks after exposure.

Brucella agglutinins developed in all pigs and reached levels of 1:100 and higher. The infections were not clinically apparent and at necropsy, nine to 16 weeks after exposure, *Br. neotomae* was not isolated, although 6 animals still had agglutinins.

The bacteriological character of *Br. neotomae*, including its infectivity for guinea pigs, was not altered by residence in tissues of swine.

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XUM

# Hemagglutination by Trypsin-Modified Infectious Bronchitis Virus

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THE CAPACITY of certain viruses to cause hemagglutination has served as a useful diagnostic and investigative method for their identification and characterization.[8] Based on the mechanism of virus-erythrocyte reaction, viruses have been classified in three general groups: (1) mumps, Newcastle disease, and influenza (MNI group), in which adsorption to specific cell receptor sites and enzymatic elution by the virus with consequent alteration of the cell surface are characteristic; (2) vaccinia, variola, and ectromelia, which are characterized by a soluble and distinct hemagglutinin separable from the virus particle; and (3) a variety of viruses including Japanese B, St. Louis encephalitis, Russian encephalitis, mouse encephalomyelitis (Theiler's GD VII strain), west Nile fever, encephalomyocarditis group, mouse pneumonia, foot-and-mouth disease, and fowl plague, in which spontaneous elution does not occur and rather exacting conditions are necessary for the reaction.

Not all viruses possess hemagglutinative activity and, with some, this has been induced by either chemical modification of the erythrocyte surface or by enzymatic treatment of the virus.[2, 15, 20]

Inhibition of hemagglutination by homologous antibody may be employed as a diagnostic procedure with many viruses. In addition to hemagglutination-inhibition by specific antibodies, a variety of nonspecific inhibitors belonging to the group of mucins or mucoproteins are present in bacterial extracts, egg albumin, allantoic fluid, serum, and other biological substrates. Inactivation of nonspecific inhibitors has been accomplished by filtration,[3] trypsinization,[11, 18] heat,[13] sodium citrate,[6] sodium periodate, and dialysis.[21] Among the nonspecific inhibitors, the Francis inhibitor[7] in serum is best delineated. It may be inactivated by influenza virus and the receptor-destroying enzyme of *Vibrio cholerae*.[1]

Infectious bronchitis virus (IBV) does not possess hemagglutinative activity.[5] The purpose of the present study was to investigate possible means of inducing hemagglutination and consequent inhibition to provide a diagnostic test for the virus.

## MATERIALS AND METHODS

One egg-adapted strain and 11 strains of early egg passage were used. These strains were from the North Central Infectious Bronchitis Virus (IBV) Repository at Michigan State University and were identified by repository code and source as follows: Code 1, 2, 3 (Hofstad 314, 33, and 104, respectively), 16 (Hipolito 66), 19–24 (6 strains from Alabama identified at the repository), 40 (MSU 586), 41 (Van Roekel, 291st chicken passage), and 42 (Beaudette B588 egg-adapted strain). The strains will be referred to by their repository code number and egg passage; e.g., 40–5: code 40, fifth egg passage, with the exception of 42. This strain has been through hundreds of passages but the exact number is unknown.

A strain (MSU 51–52–308) of Newcastle disease virus (NDV) was used for certain comparative studies of hemagglutinative activity.

Normal allantoic fluid (NAF) was used as a control.

All viruses were cultivated in embryonating chicken eggs inoculated via the allantoic cavity. Nine-day embryos were used except for IBV 42 and NDV which were inoculated in 10-day embryos. Normal and virus-infected allantoic fluids were collected from 12-day embryos. At the time of collection, fluid from all eggs from one inoculum was pooled, distributed into an appropriate number of vials and stored at −30 C. At the time of use, the fluid was thawed, centrifuged at 4 C. for 20 minutes at 3,000 r.p.m., and the clear supernatant fluid was removed for use in the tests. Samples were not refrozen.

Titrations to determine infectivity and neutralization by serum antibody were performed by using serial tenfold dilutions of virus and 5 embryos per dilution.[4] End points were determined by the

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method of Reed and Muench [16] and expressed as the infective dose $_{50}$ (i.d.$_{50}$) and the infectivity dose $_{50}$ neutralization index (i.d.$_{50}$n.i.) to log 10. Mortality and gross lesions were the criteria for infectivity of strains other than 42, for which mortality alone was the criterion.

Hemagglutination (HA) tests were performed by the standard procedure using 0.25-ml. portions each of serial twofold dilutions of virus, Difco* HA buffer, and a 0.5 per cent suspension of chicken erythrocytes. After incubation at room temperature, for 45 to 60 minutes, hemagglutination was determined by observing the pattern formed by the cells. The titer, expressed as HA units, was the reciprocal of the highest dilution of virus in which hemagglutination was complete.[4]

Hemagglutination-inhibition (HI) tests were performed by the standard *alpha* and *beta* procedures.[4, 17]

Trypsin (1:250 or 2 x crystalline, salt free), chymotrypsin, chymotrypsinogen, papain, pepsin (1:10,000), rennin, egg white trypsin inhibitor, and soybean trypsin inhibitor (5 x crystalline) were used for the basic studies of induction of hemagglutination by modification of IBV. Numerous amino acids, peptones, and colloids were also used and will be referred to in the appropriate portion of the text.

## RESULTS

*Modification of Infectious Bronchitis Virus with Trypsin.*—Preliminary investigations showed that when a Seitz EK filtrate of a 1 per cent aqueous suspension of trypsin (1:250) was added to IBV-infected allantoic fluid and incubated, the mixture caused hemagglutination of chicken erythrocytes.

To determine the effect of time, temperature, and ratios of IBV to trypsin (T) and the influence of NAF, 1 ml. of T was added to 1, 2, 3, or 9 ml. of IBV and NAF. The mixtures were incubated at 4 C., 23 C., and 37 C. At certain time intervals for as long as 720 minutes, portions of the mixtures were removed and used in the HA test. Controls consisted of T alone or in combination with buffer. Hemagglutination also occurred with the controls and NAF, but the maximum and most uniform results (HA titers ranging from 1,280 to 5,120 and occasionally higher) were obtained with samples containing 1 ml. of T to 2 ml. of IBV which had been incubated at 37 C. for three hours. This combination was selected for further study.

To inhibit the induced HA activity, the T-IBV mixtures were heated at 100 C. for two minutes at the end of the incubation period. This treatment caused precipitation of proteins and the mixtures were valueless for the test. The supernatant fluid did not possess HA activity. When 1 ml. of a 1 per cent aqueous solution of egg white trypsin inhibitor (ETI) was added to T-IBV and T-NAF at the and of the incubation period and to the controls, the HA activity of the controls and T-NAF was eliminated. With the egg-adapted strain, IBV 42, HA activity was eliminated in most instances and was greatly reduced in others. Some tests showed titers as high as 320. Slight, if any, reduction of titers was evidenced with the early egg passage strains of IBV. Identical results were obtained when soybean trypsin inhibitor (STI) was used under the same conditions.

Based on these findings, the procedure for the trypsin-induced infectious bronchitis (TIB) HA test (TIB-HA) was standardized as follows: (1) add 1 ml. of 1 per cent T to 2 ml. of IBV, (2) incubate for three hours at 37 C., (3) add 1 ml. of 1 per cent ETI, (4) prepare serial twofold dilutions and perform the test. The pH of this mixture is about 7.2 which is within the range of stability of influenza hemagglutinin (pH 6.0 to pH 8.0). Chicken erythrocytes are spontaneously agglutinated or lysed at greater extremes of pH.[12] The ingredients were maintained free of bacteria until the addition of ETI, which was not filtered, as this resulted in diminished inhibitory activity by adsorption. Egg white trypsin inhibitor was selected over STI because of economy and greater solubility. Typical results with the TIB-HA test are shown (table 1).

Crystalline trypsin (2 x T) could be used in the test but not substituted for T in equal concentration, as hemagglutination did not occur when a 1 per cent solution 2 x T was employed. At the optimum concentration, the following titers were obtained when 1 ml. of 0.25 per cent 2 x T and 0.25 ml. of 1 per cent ETI were used: IBV 40–5, 1,280 to 5,120, and IBV 42, 160. Hemagglutination did not occur with NAF or the controls.

A further investigation was undertaken using chymotrypsin and chymotrypsinogen. Since no inhibitor for chymotrypsin was available, 2 x T was used to activate chymotrypsinogen for conversion into chymotrypsin. Inhibition of chymotrypsin was controlled by inactivating 2 x T with ETI.

* Difco Laboratories, Inc., Detroit, Mich.

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Chymotrypsin caused agglutination of cells alone to a titer of 640 as contrasted to the inability of chymotrypsinogen to induce hemagglutination. When either 0.25 ml. or 1 ml. of a 1 per cent solution of chymotrypsinogen, activated by 0.1 ml. of 1 per cent 2 x T for three hours at 37 C., was added to 2 ml. of IBV or NAF and inhibited by 0.1 ml. of 1 per cent ETI, the titers were uniformly 1,280 for IBV 40–5 and 160 for IBV 42. The NAF samples were negative.

*Other Enzymes.*—Seitz EK filtrates of aqueous solutions of 1 per cent pepsin, rennin, and 0.5 per cent papain did not induce hemagglutination by IBV 40–5, 42, or NAF as mixed for the test without the addition of ETI. These enzymes were without effect on the titer of NDV when employed in the same manner as for IBV and NAF.

*Species of Erythrocytes.*—The test could be performed with chicken erythrocytes but not with canine, feline, equine, ovine, bovine, lapine or human type O cells.

*Effect of Trypsin and Cellular Adsorption of Virus on Viral Infectivity.*—To 2 ml. of a 25 per cent suspension of cells, 1 ml. of T was added and the mixture incubated at 37 C. for three hours. After the addition of 1 ml. of ETI, the cells were washed in saline solution through three cycles of centrifugation. These cells and normal cells were used with T-modified IBV 40–5, 42, NAF, and untreated IBV 40–5.

Hemagglutination did not occur with the T-modified cells, virus, or NAF. Only T-modified IBV 40–5 reacted with the normal cells (HA titer, 5,120).

Strain 40–5, which was capable of retaining a titer of $i.d._{50}^{6.2}$ after three hours at 37 C., was processed for the test. After the standard incubation period of three hours with T at 37 C. and the addition of ETI, the titer was $i.d._{50}^{4.8}$. To 1 ml. of the TIB mixture, 1 ml. of cells was added and the mixture was incubated at 23 C. for ten minutes. After centrifugation at 3,000 r.p.m. for ten minutes at 4 C., the cell-free supernatant fluid had a titer of $i.d._{50}^{4.3}$.

*Thermostability.*—Strain 40-5 was heated at 56 C. and at 30-minute intervals samples were removed for use in the test. The initial titer of 640 was retained for as long as 150 minutes, but at 180 minutes the titer was 160. As measured by infectivity for embryos, this strain resists 56 C. for as long as 120 minutes.

Strain 42 and NAF, subjected to the same treatment as 40–5, showed no evidence of hemagglutinative activity.

Several strains at various embryo passage levels were trypsinized, stored at −30 C. for different periods, and the titers compared prior to and after storage. There was no uniformity of results and, in some instances, the HA activity of the virus was markedly reduced after storage for one day.

*Adsorption and Elution of Virus.*—With the TIB-HA test for IBV, elution is not a characteristic finding as with the HA test for NDV. When the test was performed with IBV 40–5 at 4 C., the pattern of cells was incomplete at 30 and 45 minutes. At 60 minutes, the titer was 1,280 but the pattern of cells was not distinct. The titer was 640 at 80 minutes with a distinct pattern of cells. Elution had occurred in all dilutions greater than 1:20 at the fourteenth hour. When the test was performed at 23 C., the titer was 2,560 at 30 through 80 minutes but, at the fourteenth hour, elution had occurred in dilutions greater than 1:320. At 37 C., the titer was 2,560 at 30 and 45 minutes and 1,280 at 60 and 80 minutes. At the fourteenth hour, elution had not occurred and some hemolysis was evident.

The TIB-HA system consisting of 1.0 ml. each of virus containing 25 HA units per 0.25 ml., buffer, and 0.5 per cent cells was dispensed in a series of tubes and incubated at 4 C., 23 C., and 37 C. All ingredients were at thermal equilibrium at the time of mixing. Immediately after mixing and at five-minute intervals for as long as 25 minutes, one sample was centrifuged at 3,000 r.p.m. for ten minutes at 4 C. The cell-free

**TABLE 1—Typical Hemagglutination (HA) Titers of Some Trypsin-Modified Infectious Bronchitis Virus (IBV) Strains**

| IBV | Source | HA titer |
|---|---|---|
| 1–7 | Hofstad 314, Iowa | 2,560 |
| 2–10 | Hofstad 33, New York | 5,120 |
| 3–6 | Hofstad 104, Iowa | 2,560 |
| 16–34 | Hipolito 66, Brazil | 1,280 |
| 19–5 | Roberts 99, Alabama | 1,280 |
| 20–10 | Roberts 603, Alabama | 2,560 |
| 21–8 | Roberts 571, Alabama | 640 |
| 23–15 | Roberts 331, Alabama | 640 |
| 24–10 | Roberts SIMS, Alabama | 2,560 |
| 40–5 | Cunningham 586, Michigan | 2,560 |
| 41–1 | Van Roekel 291, Massachusetts | 2,560 |
| 42–? | Beaudette B588, New Jersey (egg-adapted strain) | 0 |
| ..... | Normal allantoic fluid | 0 |

supernatant fluid was removed and tested for hemagglutinative activity.

At 23 C. and 37 C., the hemagglutinin was completely and immediately adsorbed to the cells as shown by lack of HA activity of the supernatant fluid from all samples. At 4 C., the HA titer of the supernatant fluid was 5 for all samples at each reaction period. Unadsorbed virus which served as the control retained the initial HA titer of 25.

To determine the rate of adsorption of IBV to cells, multiple increments of virus containing 512, 1,024, 1,536, and 2,048 HA units per 0.25, 0.5, 0.75, and 1.0 ml., respectively, were added to 0.25 ml. of cells and the volume of each sample adjusted to 1.25 ml. with appropriate amounts of buffer. After incubation at 23 C. for 45 minutes, the samples were centrifuged at 3,000 r.p.m. for ten minutes at 4 C. and the supernatant fluid tested for HA activity. The titers of the supernatant fluids from the samples originally containing 512, 1,024, 1,536, and 2,048 HA units were 10, 20, 40, and 80, indicating rates of adsorption of 98, 98, 97, and 96 per cent, respectively.

*Hemagglutination and Infectivity of Infectious Bronchitis Virus for Chicken Embryos.*—Strains 40 and 41 were used to compare HA titer to infectivity as measured by mortality and gross pathological alterations of embryos used for isolation of IBV. As shown (table 2), there is a direct relationship between HA titer and infectivity. This is emphasized by the parallelism of the first three passages of strain 40 in embryos and the three passages following recovery of the virus from chickens inoculated with the fifth embryo passage material. The relationship was shown with strain 41 in the first embryo passage.

**TABLE 2—Hemagglutination (HA) and Embryo Infectivity of Infectious Bronchitis Virus (IBV) 40 and 41**

| IBV | Embryo response* | HA titer |
|---|---|---|
| 40–1 | 2/10 | 0 |
| 40–2 | 2/10 | 0 |
| 40–3 | 7/10 | 5,120 |
| 40–5–1C–1† | 1/10 | 0 |
| 40–5–1C–2† | 2/10 | 0 |
| 40–5–1C–3† | 8/10 | 2,560 |
| 41–1 | 7/10 | 5,120 |

* Mortality and gross pathological alterations of embryos.
† IBV 40–5 was used to infect 3-week-old chickens. The IBV was recovered from lung and tracheal suspensions and inoculated into eggs. The last number indicates egg passage.

*Optical Density, Hemagglutination, and Infectivity.*—When ETI was added to T-modified IBV other than strain 42 or NAF, the mixture became turbid within ten minutes. This effect was not evident when 2 x T was used for modification of the virus. Several strains of IBV were prepared for the test and the optical density was determined at a wave length of 500 mμ with a spectrophotometer.* As shown (table 3), there was a relationship between optical density and HA titer.

**TABLE 3—Optical Density and Hemagglutination (HA) Titer of Some Infectious Bronchitis Virus (IBV) Strains**

| Strain | HA titer | Optical density (500 mμ) |
|---|---|---|
| 1–7 | 640 | 0.24 |
| 2–11 | 5,120 | 0.62 |
| 3–6 | 1,280 | 0.32 |
| 40–5 | 2,560 | 0.42 |
| 40–5 | 2,560 | 0.48 |
| 41–1 | 2,560 | 0.50 |
| 42 | 0 | 0.0 |
| NAF | 0 | 0.0 |

To further test this relationship, the HA titer and optical density of strain 40–5 were compared with undiluted and serial two-fold dilutions of the virus. In addition, the effects of these dilutions on embryo infectivity prior to T-modification of the virus were examined. The results of these tests are presented (table 4). There was a relationship between HA titer and optical density at the various dilutions, but it was not as direct as the regression line for the effect of dilution on embryo infectivity.

*Hemagglutination-Inhibition Tests.*—Normal and anti-IB chicken serums were used for hemagglutination-inhibition (HI) tests by the *alpha* and *beta* (10 HA units) procedures with IBV prepared for the TIB-HA test. Identical HI titers were obtained with the negative and positive serums with the respective tests.

Serum dilutions as high as 1:25 were employed for the *alpha* procedure but complete inhibition always occurred. Using the conversion formula

$$\frac{\text{virus titer} \times \text{dilution of serum}}{\text{serum titer}} = \text{HI titer}$$

the HI titer was always five times the HA titer of the virus, e.g., HA 1,280, HI 6,400; HA 2,560, HI 12,800. The HI titer of a se-

* Beckman Model B Spectrophotometer, manufactured by Beckman Instruments, Inc., South Pasadena, Calif.

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rum was not constant but varied from test to test according to the HA titer of the virus preparation used.

With the *beta* procedure, inhibition always occurred through the 1:1,280 dilution of serum for an HI titer of 12,800 when ten HA units were used. This constant HI titer

**TABLE 4—Optical Density, Hemagglutination (HA) Titer, and Infectivity of Infectious Bronchitis Virus (IBV) 40-5**

| Dilution | HA titer | Optical density (500 mμ) | Embryonic infectivity prior to treatment (log 10/0.1 ml.) |
|---|---|---|---|
| Undil. | 2,560 | 0.40 | 7.0 |
| 1:2 | 640 | 0.30 | 6.7 |
| 1:4 | 20 | 0.185 | 6.4 |
| 1:8 | 10 | 0.10 | 6.1 |
| 1:10 | 0 | 0.078 | 6.0 |
| 1:16 | 0 | 0.05 | 5.8 |

was obtained regardless of whether virus preparations having HA titers of from 320 through 10,240 were diluted to contain ten HA units for the test.

In an attempt to inactivate possible nonspecific inhibitors, normal serum was subjected to the following treatments: (1) 56 C. for 30 minutes; (2) 62 C. for 20 minutes;[13] (3) addition of an equal volume of 3.81 per cent sodium citrate to undiluted serum;[6] (4) filtration through Seitz EK pad;[3] (5) combination of equal volumes of serum and filtrates of *Clostridium welchii* types A, C, and D at 23 C. for one hour;[14] (6) ether extraction at 23 C.; (7) 62 C. for 20 minutes and then treated with T and ETI as in the HA test; (8) 2 x T and ETI or STI as in the HA test; (9) T and ETI as in the HA test; and (10) combination of T-modified IBV and cells prior to the addition of serum.

With one exception, all treatments were without effect on the inhibitory properties of normal serum. The exception was obtained only with the *beta* procedure using serum that had been heated at 62 C. for 20 minutes and then treated with T and ETI as for virus in the HA test. The IBV 40–5 (HA 320) yielded an HI titer of 800 with positive serum and 50 with normal serum. The IBV 40–23 (HA 640) had an HI titer of 800 with positive serum and 0 with normal serum. The differential inhibition by positive and normal serum detected by the *beta* procedure could not be duplicated through numerous repeated tests with these and other strains and no reason can be advanced at this time as to their occurrence.

Using the *alpha* procedure, the HI titers of the positive and normal serums were 1,600 for IBV 40–5 and 3,200 for IBV 40–23. Several other *alpha* procedure tests with IBV 40–5 (HA 2,560) yielded uniform HI titers of 12,800 with positive and negative serums, which illustrates the previous experience with unheated serum of the HI titer being five times greater than the HA titer of the virus. *Beta*-procedure HI titers were 6,400 for the same serum. The lessened inhibition of the heated and trypsinized serum by the *beta* procedure, through the 1:640 dilution of serum in this case as compared to the 1:1,280 dilution with unheated serum, was the only other difference noticed between treated and untreated serum. This difference may possibly have been due to technical manipulations, but it was constant for all *beta* procedure tests.

To ascertain the possible inhibitory effect of components of the HA test, untreated NAF, IBV 40–20 and IBV 42, and ETI and STI were diluted 1:5 and substituted for serum in the *alpha* procedure with T-modified IBV 40–5. Newcastle disease virus was used as a comparative control. The results of these tests are shown (table 5).

**TABLE 5—Hemagglutination-Inhibition (Alpha Procedure) with Agents Other than Serum**

| Hemagglutinin | HA titer | Inhibitor | HA titer after combination with inhibitor |
|---|---|---|---|
| NDV | 1,280 | ..... | ..... |
| IBV* | 2,560 | ..... | ..... |
| NDV | 1,280 | NAF | 160 |
| IBV | 2,560 | NAF | 0 |
| NDV | 1,280 | IBV–42 | 80 |
| IBV | 2,560 | IBV–42 | 0 |
| NDV | 1,280 | IBV 40–20 | 80 |
| IBV | 2,560 | IBV 40–20 | 0 |
| NDV | 1,280 | ETI | 1,280 |
| IBV | 2,560 | ETI | 0 |
| NDV | 1,280 | STI | 1,280 |
| IBV | 2,560 | STI | 2,560 |

HA = hemagglutination; NDV = Newcastle disease virus; IBV = infectious bronchitis virus; NAF = normal allantoic fluid; ETI = egg white trypsin inhibitor; STI = soybean trypsin inhibitor.
* Strain 40–5.

Normal and IBV-infected allantoic fluids completely inhibited hemagglutination by T-modified IBV, and caused eight- and 16-fold reductions, respectively, of the HA activity of NDV.

Inhibition of IBV-HA by ETI but not by STI indicated that hemagglutination was not a result of activity of possible residual trypsin in the HA test system. Egg white

trypsin inhibitor, which is ovomucoid and represents one of the Francis type inhibitors, and STI were not effective against NDV hemagglutinin.

To provide another indication of the distinction of hemagglutination, titrations were made using T alone and the HA test. After hemagglutination had occurred, 0.25 ml. of ETI (1:5) was added to each tube which was then shaken to resuspend the cells. The cells which had been agglutinated by T were not reagglutinated, whereas those which had been agglutinated by T-modified IBV were reagglutinated.

The supernatant fluid from *Clostridium welchii*, type A, was diluted 1:5 and tested for inhibition of the TIB-HA test.[19] Hemolysis occurred but agglutination was apparent through the 1:180 dilution of the virus.

*Hemagglutination by Peptones, Polypeptides, and Amino Acids.*—Hemagglutination by trypsinized IBV was the stimulus for investigation of the effect of some of the products of tryptic hydrolysis of other proteins. When serial twofold dilutions of 1 per cent aqueous solutions of trypticase, tryptone, and tryptose were substituted for virus in the standard HA test,[4] the following titers were obtained: trypticase, 400; tryptone, 800; and tryptose, negative. The HA titers are expressed as the reciprocal of the grams per milliliter of solution; e.g., a 1 per cent solution contains 1 Gm. per 100 ml. and if only the undiluted sample caused hemagglutination the titer is expressed as 100. If the 1:4 dilution of the 1 per cent solution agglutinated cells, the titer would be 400. After the addition of 1 per cent ETI at the rate of 0.1 ml./1 ml. of solution the following titers were obtained: trypticase, 100; tryptone, 800; and tryptose, negative. Hemagglutination by trypticase and tryptone, but not by tryptose, serves to emphasize the range of hemagglutinative agents and the question of the specificity of the T-induced IBV hemagglutinin.

Solutions of L-arginine, glycine, L-lysine, L-methionine, L-phenylalanine, L-serine, and L-tyrosine, alone or in combination, exhibited no hemagglutinative activity.

An aqueous solution of trypticase, glycine, L-arginine, L-lysine, L-methionine, and L-serine, each at 1 per cent final concentration, had an HA titer of 6,400. Expressed as the reciprocal of grams per milliliter of the six ingredients, the titer would be reduced to 1,067. Since the amino acids in themselves do not cause hemagglutination, the titer based on the weight of trypticase alone is 6,400.

Arginine and lysine represent the points of initial tryptic activity. A solution of 1 per cent each of tryticase, L-arginine, and L-lysine had an HA titer of 400 on the basis of tryticase alone, but a titer of 133 expressed as the reciprocal of grams per milliliter of the three ingredients.

From the titers based on the weight of trypticase, it is seen that the addition of the amino acids other than arginine and lysine enhanced hemagglutinative activity, whereas arginine and lysine did not have this effect.

## DISCUSSION

Modification of certain properties of IBV by trypsin indicates that arginine or lysine or both may be present in the molecular structure of the viral protein, as trypsin cleaves the peptide bonds involving the carboxyl groups of these amino acids. The spatial configuration of the protein molecule and its position in the virus particle may permit formation of an enzyme-substrate complex.

Interpretation of the possible mechanism of reaction of the TIB-HA test must be primarily directed to the components in both normal and virus-infected allantoic fluid. Urine contains a trypsin inhibitor (UTI) and, since the components of urine are contained in NAF, it is likely that the introduction of T forms an enzyme-inhibitor complex, T-UTI, which agglutinates erythrocytes. It may be that T-UTI is either adsorbed to the cell or dissociated, with subsequent adsorption of T alone to the cell. The inhibitory effect of ETI on hemagglutination tends to support the theory of adsorption of T-UTI to the cell surface. If there were dissociation of T-UTI, it could be assumed that T then reacted with ETI since hemagglutination did not occur, which is characteristic of this combination. In the absence of the turbidity that accompanies T-ETI, it may be concluded that the initial T-UTI complex reacts with ETI to form a soluble complex, ETI-T-UTI, which is not adsorbed on the cell surface and does not have hemagglutinative capacity.

Trypsinized IBV represents a true enzyme-substrate complex and the resultant modification of the virus provides a product which is adsorbed to the cell surface and causes hemagglutination. In the test, the

XUM

turbidity of the T-IBV-ETI mixtures is related to HA titer and reflects virus concentration. Turbidity is also associated with T-ETI, but hemagglutination does not occur. It is postulated that T has an affinity for both IBV and UTI, but preferentially reacts with IBV, is released, and reacts with more virus. The continuity of reaction is disrupted by the addition of ETI and the formation of T-ETI which is evidenced by turbidity of the mixture.

It may be concluded that hemagglutination exhibited by the test is either the product of adsorption of modified virus or of soluble degradation products derived from the virus through tryptic digestion or a combination of both. The cell serves as an indicator of the reaction and participates only with T-modified IBV. Trypsin-modified cells do not produce hemagglutination with untreated IBV. No enzymic activity of the virus itself is involved in hemagglutinative activity since heat-inactivated virus participates in the test. Denaturation of virus protein by excessive heat diminishes the HA titer which may be an indication of alteration of the virus particle so that sites previously reactive with T are modified or destroyed.

Consideration of the ratio of viral infectivity to HA titer on the most commonly occurring titrations (i.d.$_{50}$ 7.2/ml., HA 2,560/0.025 ml.) on a unity basis would indicate 1,544 infective units per HA unit. This does not take into consideration that inactive virus particles may be modified by T to serve as hemagglutinative units. Approximately $7.5 \times 10^6$/0.25 ml. cells are used in the test and if the total is considered reactive, the one virus particle must serve as hemagglutinin for 4,858 cells. It would be physically impossible for one particle to serve as the entire hemagglutinative bond for this number of cells. That the virus particle is associated in part with HA activity is evidenced by the reduction of infectivity following combination of virus with cells.

The minimal reaction or complete absence of hemagglutination with the egg-adapted strain of IBV may be an expression of change of physical or chemical structure of the viral protein associated with the decrease and loss of pathogenicity and immunogenicity of the virus for chickens which accompanies egg-adaptation of the virus.

The IBV hemagglutinin differs from that of influenza hemagglutinin which is completley adsorbed to cells at 4 C., whereas maximum adsorption of IBV hemagglutinin occurs at 37 C. The HA reaction also differs from that of the MNI group of viruses in that spontaneous elution does not occur. Adsorption of influenza virus appears to be of a physical nature but that of IBV is more representative of chemisorption. Another difference is that cells are not reagglutinable by the same strain of influenza virus or those lower on the receptor gradient scale.[9,10] With IBV hemagglutinin, cells are reagglutinable.

Specificity of the IBV hemagglutinin has not been demonstrated by inhibition with anti-IB serum.

So far as known, this is the first evidence presented on the participation of IBV in an hemagglutination system and initial explaation of the possible chemical configuration of IBV protein.

## SUMMARY

1) Infectious bronchitis virus modified by trypsin and the subsequent addition of either egg white trypsin inhibitor or soybean trypsin inhibitor contained an active hemagglutinating agent.

2) The completely egg-adapted strain of the virus did not react to as great an extent in this system as did less well-adapted strains.

3) A 98 per cent reduction in embryo infectivity of the virus accompanied trypsin modification.

4) The rate and degree of adsorption of the hemagglutinin varied directly with temperature. Elution varied indirectly with temperature.

5) The hemagglutinin was probably present in two fractions: one in close association with the virus particle, and the second, which constituted the greater part, was distinct from the virus particle.

6) Hemagglutination varied directly with virus concentration.

7) There was a direct relationship between optical density of the trypsinized virus with egg white trypsin inhibitor and hemagglutination titer.

8) Hemagglutination was readily inhibited by negative and anti-infectious bronchitis serum, ovomucoid, and normal and virus-infected, allantoic fluid.

9) The infectious bronchitis virus hemagglutinin was distinct from that of other viruses which have been demonstrated to have hemagglutinative activity.

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# Experimental Ornithosis in Herons and Egrets

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SINCE THE REPORTS of ornithosis in turkeys in 1951 [4] there has been considerable speculation as to how the ornithosis agent is introduced into turkey flocks. Only a few flocks are known to be involved yearly. This sporadic nature indicates the presence of a vector or carrier animal as yet unidentified. Ecological studies of known infected flocks in Texas revealed that the turkeys in each instance were drinking from surface water.[3]

Because herons and egrets frequent water and are migrating during the time of these outbreaks, they have been suspected as possible carriers of infection. In the fall of the year, the summer hatchings wander great distances from the nesting site, possibly because of overcrowding on their breeding grounds. Thus, an infected heron or egret may reach an isolated flock of turkeys that are drinking from surface water. With each intensive cold wave, the birds move south from pond to pond and frequently attempt to land among flocks of white turkeys.

During the late spring and throughout the summer, herons and egrets congregate at the breeding grounds. Often as many as ten species of birds nest in the same rookery. Commonly, three or more species nest in the same tree. This enhances the possibility of an infected bird spreading the disease among several species.

In 1951, Rubin *et al.*[5] isolated a highly virulent strain of ornithosis from nestling egrets from an island near the Louisiana coast.

Experimental work by Davis *et al.*[2] indicated that egg transmission is not a factor in the epizootology of the disease in turkeys. Also, the history of the disease would not indicate egg transmission because it occurred in alternate years instead of annually and has usually been in semimature or mature birds. In experimental infection, mortality is higher in younger turkeys.

In view of the findings by previous workers, it seems desirable to determine the effect of ornithosis in herons and egrets, to observe the interval of latent infections, and to assess the adequacy of the indirect complement-fixation (ICF) test as an indication of the disease of herons and egrets.

## EXPERIMENTAL PROCEDURE

Sixteen adult herons and egrets were collected from a rookery and brought into a security-type laboratory. For the first few days these birds were force-fed chopped fish, and then were maintained on a chopped fish diet which was placed in a pan of water. Their serological titers were negative by the ICF test. The ICF test was selected because a high percentage of avian serums do not fix complement. The birds were inoculated with 0.25 ml. of ornithosis-infected amnioallantoic fluid intratracheally. The Jo strain [1] originally isolated from turkeys was used for this experiment. Blood samples were taken at intervals after inoculation of the birds and serological titers determined by the ICF test.

Once a week cloacal swabs were obtained from each bird and the material was suspended in nutrient broth containing 25 mg. of dihydrostreptomycin per milliliter. The suspension was then centrifuged at 5,000 r.p.m. for ten minutes to eliminate contaminating bacteria. The supernatant fluid was inoculated into five 7-day chicken embryos by way of the yolk sac. At least two blind passages were made before discarding a sample as negative.

The agents isolated in eggs were inoculated into mice using the intraperitoneal route. Lesions, deaths, and presence of Levinthal-Coles-Lillie (LCL) bodies in mice were used as criteria for identification of ornithosis virus. The isolation which was obtained the longest period after experimental infection of herons was inoculated into mice intraperitoneally and intracerebrally and into 5-week-old turkeys intratracheally to determine the pathogenicity and to demonstrate LCL bodies. The herons and egrets that died were necropsied and virus isolation was attempted by egg inoculation and intraperitoneal mouse inoculation.

## RESULTS

The serological titers of infected herons and egrets at various intervals are shown (table 1). These birds had titers compara-

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ble to those of turkeys inoculated with the same strain of virus. All birds inoculated developed a titer except 2 that died five and 13 days after inoculation.

**TABLE 1—Titers of Herons and Egrets Inoculated with Ornithosis Virus**

| Bird No. | Type | Preinoc. | Postinoculation day 17 | 25 | 46 |
|---|---|---|---|---|---|
| 455 | Snowy egret | 0 | 8 | 2 | 4 |
| 456 | Snowy egret | 0 | 4 | 8 | 16 |
| 457 | Little blue heron | 0 | 0 | 0 | 2 |
| 458* | American egret | 0 | .... | .... | .... |
| 459 | Snowy egret | 0 | 16 | .... | .... |
| 461 | Snowy egret | 0 | 64 | 32 | 16 |
| 462* | Little blue heron | 0 | .... | .... | .... |
| 463 | Snowy egret | 0 | 4 | 0 | .... |
| 464 | Snowy egret | 0 | 32 | 8 | 0 |
| 465 | Snowy egret | 0 | 8 | 2 | 2 |
| 466 | Snowy egret | 0 | 256 | 64 | .... |
| 467 | Snowy egret | 0 | 32 | 4 | .... |
| 468 | Snowy egret | 0 | 8 | 4 | 4 |
| 470 | Snowy egret | 0 | 16 | 4 | 2 |

* 458 and 462 died 13 and 5 days postinoculation, respectively.

The positive isolations obtained in eggs and those that subsequently proved positive in mice are summarized (table 2). The agent isolated from bird 470 on day 67 was inoculated into ten 4-week-old turkeys with a mortality of 60 per cent by the tenth day postinoculation. Turkeys alive at the tenth day were killed for necropsy. All had typical lesions of ornithosis and the virus was recovered. The agent isolated from bird 470 on day 67 killed mice three days after intracerebral inoculation; LCL bodies were demonstrated on microscopic examination. Deaths during the experiment, lesions observed, and virus isolations are shown (table 3).

**TABLE 2—Virus Isolation at Various Intervals After Inoculation with Ornithosis Virus**

| Bird No. | Type | Days postinoculation 10 | 17 | 25 | 60 | 67 |
|---|---|---|---|---|---|---|
| 455 | Snowy egret | .... | .... | .... | .... | .... |
| 456 | Snowy egret | .... | .... | E | .... | .... |
| 457 | Little blue heron | .... | .... | .... | M | .... |
| 458 | American egret | .... | .... | .... | .... | .... |
| 459 | Snowy egret | M | .... | .... | .... | .... |
| 461 | Snowy egret | .... | .... | .... | .... | .... |
| 462 | Little blue heron | .... | .... | .... | .... | .... |
| 463 | Snowy egret | .... | .... | .... | .... | .... |
| 464 | Snowy egret | .... | M | .... | .... | .... |
| 465 | Snowy egret | .... | .... | .... | .... | M |
| 466 | Snowy egret | .... | .... | .... | .... | .... |
| 467 | Snowy egret | .... | .... | .... | .... | .... |
| 468 | Snowy egret | .... | .... | .... | .... | .... |
| 470 | Snowy egret | .... | .... | M | .... | M |

E = isolation in eggs. M = mortality in mice and demonstration of Levinthal-Coles-Lillie bodies.

## Discussion

Herons and egrets experimentally inoculated with a highly virulent strain of ornithosis showed no sign of infection. They did retain the virus and shed it intermittently for as long as 67 days. This occurrence under natural conditions suggests that a heron or egret could move vast distances from its source of infection, remain apparently healthy, and transmit the disease by its droppings. Evidently the agent is harbored in the cloaca, because birds that died after 13 days had no virus in their spleens. Birds dying after 13 days apparently died from a lack of nutrition or from fighting, which was difficult to control.

**TABLE 3—Lesions and Virus Isolation from Birds Which Died During Study**

| Bird No. | Postinoculation day of death | Virus isolation | Lesions |
|---|---|---|---|
| 458 | 13 | + | Pericarditis |
| 459 | 21 | — | None |
| 462 | 5 | + | None |
| 463 | 41 | — | None |
| 466 | 31 | — | None |
| 467 | 25 | — | None |
| 469 | 7 | — | None |

The possibility of reinfection from environment was not eliminated, but in no case did birds develop a serological response indicative of reinfection. In most cases, when virus was isolated the titer had reached its peak and was receding.

There seems to be an association between the highly virulent strains of ornithosis of these water fowl and the one isolated by Rubin *et al.*[5] Most of the strains isolated from turkeys where herons, egrets, and sea gulls could be incriminated are the highly virulent type (Texas, New Jersey, and Oregon), whereas other strains are mild (Minnesota, Ohio, and California).

Dihydrostreptomycin at a level of 25 mg. per milliliter of suspended cloacal contents and centrifugation were not completely effective in reducing bacterial contamination.

## Summary

Herons and egrets were experimentally infected with a turkey strain of ornithosis. The birds remained apparently normal and developed a serological titer as indicated by the indirect complement-fixation test. Virus was obtained from cloacal swabs for as long as 67 days postinoculation.

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# A Discussion of Synonymy in Avian and Mammalian Hematological Nomenclature

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THE SAME CELL TYPES are generally represented in the blood of all vertebrates and, judging from the review of comparative hematology by Jordan,[7] the cellular processes of hematocytogenesis follow the same basic pattern in all classes of this subphylum. For a full understanding of the processes of hematology in any one species, there must be a broad understanding of the subject derived from an adequately detailed comparative hematology.

Practically the sum total of effort in the past has gone into a study of hematology for one species, man. It is natural, therefore, that the terminology put forth from time to time should be influenced by and agree with the observations made on man and other mammals.

Had the terminology for vertebrate hematology been developed first upon the basic principles derived from a detailed comparative study of all classes of vertebrates, we would today probably have a terminology that could be readily and widely accepted. Upon the framework of such a basic scheme of hematological processes there could be modifications in terminology to fit peculiarities of individual orders, families, genera, and species, if this should be necessary. Unfortunately, the "tail wags the dog," and we are at present attempting to apply to all other species a terminology built primarily for man.

An additional step has been made toward an adequate comparative hematology by the preparation of the "Atlas of Avian Hematology,"[10] but we still lack sufficient detailed information on the hematology of reptiles, amphibians, and fishes. These are projects in basic research and, if we can judge from the past, will lag behind our efforts to solve other problems that have immediate application to man and his domesticated animals. Nevertheless, a full comparison of our terminology for just two classes—mammals and birds—should help to clear the ground for more critical assessment of new knowledge as it becomes available.

## ERYTHROCYTE SERIES

The rather wide separation in mammalian * and avian * terminology [1] for red cell development is not surprising in view of the fact that mammals differ from birds as well as from all other classes of vertebrates, as their mature erythrocytes are devoid of nuclei.

If the full developmental span of the avian erythrocyte were equivalent to only the nucleated phases of mammalian development, there would be no problem. However, it is rather certain from the similarity in the maturation processes of reticulocytes in birds and in mammals that the developmental span of avian erythrocytes is homologous to the entire developmental span of mammalian erythrocytes, not just part of it.

In the A.M.A.† terminology for mammals,[2-5] the separation of erythrocyte development into named stages is based largely on nuclear structure, whereas in birds the separation of stages following the erythroblast is based largely on color reactions of the cytoplasm. Actually, both parts of the cell are important in defining and delimiting the various stages of development, and both have been utilized by Lucas and Jamroz [10] in describing the stages in the erythrocyte series for the chicken.

Some asynchronism between nuclear and cytoplasmic maturation can be observed by anyone who has studied erythrocytogenesis, and it is this asynchronism that has led investigators to select only one or two criteria for naming of developmental stages. The question of importance seems to be: How serious is the confusion that would

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* "Mammalian" and "avian" are terms used frequently throughout this paper, but it is with the awareness that most mammalian hematology is based on man and most avian hematology on the chicken.

† In the text, the series of reports by the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs has been designated as A.M.A. terminology. In the literature cited, these papers are listed under the authorship of the Committee on Nomenclature (1948, 1949a, b, c).



XUM

result from the use of multiple criteria for naming of stages in normal development? Asynchronism probably is not as great as we have been led to believe, because we so often focus our attention on the atypical and abnormal. It would seem that the asynchronism associated with some disease conditions could be handled by the use of modifying terms.

Before pursuing this line of thought, it is necessary to examine the terms that have been used in avian and mammalian hematology, and especially to associate morphological entities with the terms that have been applied by various investigators.

**Equivalent Terms for Avian and Mammalian Erythrocytogenesis**

| Avian (R.P.L.*) | Mammalian (A.M.A.) |
|---|---|
| Early erythroblast | Rubriblast |
| Late erythroblast | Prorubricyte |
| Early polychromatic erythrocyte | Rubricyte |
| Mid-polychromatic erythrocyte | |
| Late polychromatic erythrocyte | Metarubricyte |
| Reticulocyte | Reticulocyte |
| Mature erythrocyte | Erythrocyte |

* R.P.L.—the terminology developed at the U.S. Regional Poultry Research Laboratory by Lucas and Jamroz.[3]

There is much merit in the A.M.A. nomenclature over other hematological nomenclatures for mammals, because it limits the suffix "blast" to the first stage in the erythrocyte line. The suffix "cyte," as used in both lists of names, designates all stages following the blast stage. If the root "rubri-" is to be retained for mammals and is to be made applicable to birds, there must be some shifts in the meaning and description for rubriblast, prorubricyte, rubricyte, and metarubricyte.

In order to proceed along these lines, it is necessary to make comparisons of definitions and actual cell structures. It is also necessary to consider some of the older terminologies used for mammals. It is even necessary to approach the problem of terminology beginning with the totipotent cell, because the names used for the earliest stages of erythrocyte development are influenced by concepts of histo- and hematocytogenesis.

Theories of hematocytogenesis largely revolve around one point: the degree of totipotency of which a particular cell is capable. Once a primordial cell during its differentiation has reached an irreversible stage of development, where it can no longer take on the characteristics of two or more cell lines, it is called a "blast" cell of that line.

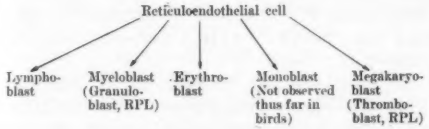
The determination of the degree of totipotency is largely a physiological problem, whereas cell identification is usually a static problem based on smears or sections. The tool of the cytologist in most of his studies must be morphological rather than physiological. This is especially true for the clinical hematologist, who daily is required to identify and name cells brought to him.

Therefore, it is recommended that the naming of cells follow cytological criteria. This procedure was used by Lucas and Jamroz.[10] An immature cell received the name of a line as soon as it could be identified with that line. An erythroblast was the earliest stage which showed cytological features that associated it with the red cell line. In avian hematology, the following types of blast cells had cytologically distinguishable features: erythroblast, thromboblast, lymphoblast, and granuloblast. In mammalian hematology, all of these blast cells look alike and often must be identified by their association with later developmental stages found on the same slide.

The greater ease of separation among different types of avian blast cells, as seen in the usual smear preparation, is due largely to the fact that erythroblasts and thromboblasts reveal nucleoli, whereas lymphoblasts and granuloblasts do not. Separation of thromboblasts from erythroblasts, and lymphoblasts from granuloblasts, often can be made even when not associated with later developmental stages, although the separation is difficult. Therefore, because the avian hematologist has some advantages over the mammalian hematologist in identifying the earliest recognizable stage of each cell line, there is a tendency in presenting the nomenclature of avian blood cells to associate an avian blast cell with a structurally similar cell taken from mammalian material that some would regard as a hemocytoblast or hemohistioblast. This is true not only for the erythrocyte line but for other cell lines as well.

However, none of the avian blast cells are equivalent to the unstimulated cells of the reticuloendothelial system. The polyphyletic theory of Undritz [15] for mammals makes the transition from the very broad reticuloendothelial cell to the specific blast

cell of each blood cell type without intermediate forms.* Undritz' scheme is as follows:

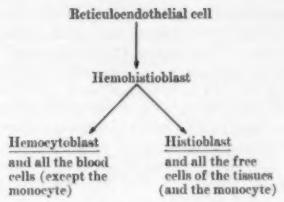



The scheme presented by Heilmeyer and Begemann [6] shows the reticular cell as the totipotent cell immediately preceding the specific blast cell for each line. This seems to be a more realistic scheme; the reticuloendothelial cell is too broad in its morphology, physiology, and potentialities of development.

The reticuloendothelial cell is not a single morphological entity; it is a whole complex of cells. Bessis [1] (p. 147) has clearly defined the reticuloendothelial system (also called reticulo-histiocytic system) as follows:

> The reticulo-endothelial system includes all cells capable of taking up "vital" stains (trypan blue, pyrrol blue, carmine, etc.). These cells fall into the following categories: (1) Reticulum cells. These are found in the reticulum of the hematopoietic organs (in the follicles and medullary cords of lymph glands, in lymph follicles, in Peyer's patches, tonsils, etc.). (2) Reticulum cells occurring in endothelium, i.e., in the walls of blood sinuses (reticulo-endothelial cells of the hematopoietic organs, the blood sinuses of the spleen, of the bone marrow, and as Küpffer cells in the blood sinuses of the spleen). They also occur in the pulmonary alveoli, in connective tissue, in the suprarenal medulla, in the pituitary, in the uterus (interstitial cells), and in some of the capillaries and lymphatics. (3) Free tissue cells. These are free reticulum cells, macrophages, clasmocytes, histiocytes, cells in the reticulum network of the skin, cells which have taken up dust and other pigments, large mononuclear cells found in the spleen, etc. (4) Blood cells in the form of monocytes. (5) Cells of the microglia attached to glial system and studied by Del Rio-Hortega (1930).

The scheme presented by Bessis [1] (p. 567) shows two intermediate stages between the reticuloendothelial cell and the specific blast cell:


Reticuloendothelial cell
Hemohistioblast
Hemocytoblast
and all the blood cells (except the monocyte)
Histioblast
and all the free cells of the tissues (and the monocyte)

Let us ignore for the present the question whether monocytes come from hemocytoblasts or histioblasts, except to say that our failure to find a blast cell for the monocytic line lends some support for a tissue origin of the monocyte line.

The reticular cell of Heilmeyer and Begemann [6] (plate 1) is similar to Ferrata's hemohistioblast as depicted schematically by Bessis [1] (fig. 368) and to some of the hemocytoblasts shown by Maximow and Bloom (pp. 100–101).[11] This cell also agrees morphologically with the primordial osteogenic cell present in avian embryonic bone marrow described by Lucas and Jamroz.[10] These authors called this cell a reticular cell as do Heilmeyer and Begemann.[6] Lucas and Jamroz found the reticular cell in impression smears from the spleen and thymus of a chick 35 days after hatching.

According to Bessis,[1] the hemocytoblast, composed of a relatively narrow, irregular rim of cytoplasm around a large nucleus that shows a delicate reticular chromatin pattern and a nucleolus, is still a totipotent cell. It is here that the main point of the difficulty seems to arise, because the avian erythroblast morphologically includes all the cytological features assigned to the hemocytoblast by Bessis. If the rubriblast pictured by Talbott *et al.*[14] is representative of the A.M.A. terminology, then it can be said that in mammals also this cell includes the features presumably assigned to a totipotent hemocytoblast.

Bessis [1] was not unmindful of some overlapping of cytological criteria in his scheme, because he states (p. 573):

> The stage of the hemocytoblast is one of brief duration. Often cells of this type can be encountered in which the nucleus is still reticular, but which already show one or more characteristics which allow the developmental line to which it belongs to be determined. The hemocytoblast which is to become an erythroblast is quite characteristic because of the intense violet basophilia of its cytoplasm, together with a feebly staining and paler internal region which gives rise to a whitish perinuclear zone. The

* Undritz [16] in a later edition states his point of view: "Many authors have assumed that the supply of new cells in the adult organism is derived, as in the embryo, from multipotent mesenchymal cells. It seems more probable that, after the initial embryonic period, each species of blood cell has its own particular stem cell (*polyphyletism*), for each cell species follows its own independent line of development, beginning with the stem cell and ending with the mature form which is released into the blood."

XUM

hemocytoblast which is to give rise to the monoblast often has some azurophil granules in its cytoplasm. The hemocytoblast which is to become a lymphocyte is distinguished by the pale staining of its cytoplasm and by the relatively small number of its nucleoli (there are often one or two, but rarely more), and by the poor staining of the nucleoli. This is a distinct characteristic, and in certain cases the nucleoli are so pale that they are scarcely visible against the background of chromatin.

These different characteristics, if unmistakable, are sufficient for the classification of the cell as a myeloblast, an erythroblast, etc., and no longer as a hemocytoblast. Nevertheless, it should be repeated that all the cells which we have described are only cells in different developmental stages. Often the nuclear and cytoplasmic characteristics are not exactly synchronous in their development (particularly in pathological conditions where the asynchronism may be the essential characteristic) and all forms of transition between the cells of the same developmental line exist. Each stage necessarily encroaches on the two neighboring stages, that which immediately precedes the stage under observation and that which immediately follows.

In the "Atlas of Avian Hematology,"[10] the term "hemocytoblast" was not applied to any cell we observed, and if the term is omitted from further consideration during the present discussion, it will aid comparisons between avian and mammalian erythroblasts.

There have been many terminologies recommended besides that proposed by the A.M.A.[2-5] The one given by Bessis[1] (p. 232) is as follows: proerythroblast; basophil erythroblast; polychromatophil erythroblast; acidophil erythroblast; erythrocyte. That given by Heilmeyer and Begemann[6] is: basophil proerythroblast; erythroblast; normoblast; reticulocyte; erythrocyte.

In both of these terminologies the suffix "-blast" is carried as long as the nucleus is retained and, if we are to be consistent with the same scheme of names, the mature, functional red blood cell of all classes of vertebrates below the mammals should be designated as some kind of an erythroblast. It is because the suffix "-blast" was carried through such a broad span of differentiation that the term "proerythroblast" was used consistently in mammalian terminology. Actually, it is a serious misnomer; anything antecedent to the erythroblast (in a strict sense) is a totipotent cell. The erythroblast (without prefix) of Heilmeyer and Begemann[6] is actually polychromatophilic and not basophilic, and it has neither nucleolus nor fine reticular chromatin pattern.

It would seem quite obvious, therefore, that there is much merit in the A.M.A. nomenclature as given for the erythrocyte series, because the suffix "-blast" is used only for the first recognizable stage, and the suffix "-cyte" for all following stages.

In the present scheme, it is suggested that the term "rubriblast" be limited to mean the first recognizable stages of erythrocyte development, where the cytoplasm is basophilic, the nucleus finely reticular, and one or more nucleoli are present. If the cytoplasm has an irregular outline, is only weakly basophilic, and the nucleocytoplasmic ratio is low, the cell is an early rubriblast. If the cytoplasm has a regular outline, concentric with the nucleus, is strongly basophilic, and the nucleocytoplasmic ratio is high, the cell is a late rubriblast.

The definitions that have been given by Osgood and Ashworth[12] for various stages of karyocytes and by the A.M.A. for various stages of rubricytes practically ignore the basophilic and acidophilic affinities of the cytoplasm. If the avian and mammalian terms for the red cell series are to be combined into a single set of terms equally applicable to both classes of vertebrates, then the present definitions and descriptions for the A.M.A. terms must be changed so that the definitions include a description of cytoplasmic staining affinity as well as nuclear structures.

In the terminology of Bessis, the adjectives "basophil," "polychromatophil," and "acidophil" are used. These can be synonymized with avian terminology better than can the A.M.A. terminology. The only objection to Bessis' terms is the use

Synonymy of Avian and Mammalian Schemes of Nomenclature

| Avian (R.P.L.) | Human (Bessis) |
|---|---|
| Early erythroblast | Hemocytoblast * |
| Late erythroblast | Proerythroblast |
| Early polychromatic erythrocyte | Basophil erythroblast |
| Mid-polychromatic erythrocyte | Polychromatophil erythroblast |
| Late polychromatic erythrocyte | Acidophil erythroblast |
| Reticulocyte | Reticulocyte |
| Mature erythrocyte | Erythrocyte |

* It was explained previously that the hemocytoblast of Bessis[1] is a totipotent cell, whereas the early erythroblast of Lucas and Jamroz[10] is considered to be the first recognizable cell of the red cell line (based on morphological features rather than physiological potentialities).

XUM

of the suffix "-blast" after the nucleolus has disapppeared.

If the prorubricyte has a nucleolus as indicated by the A.M.A. nomenclature, then it should be called a "-blast" instead of a "-cyte."

As far as our studies on avian blood are concerned morphologically, a rubriblast and a prorubricyte are both erythroblasts; they are merely the early and the late phases of the erythroblast stage. The former is of relatively rare occurrence; the latter is the cell commonly seen in bone marrow.

It has already been indicated that some names of the A.M.A. nomenclature would need to be changed and that some definitions of the terms that are retained will need to be modified and enlarged.

As a starting point for further discussion, let us present a compromise terminology that seems acceptable for all vertebrates, including birds and mammals.

**A Proposed Terminology of Erythrocytogenesis Applicable to All Classes of Vertebrates Compared with That Now in Use for Birds and for Mammals**

| Avian (R.P.L.) | Mammalian (A.M.A.) | Proposed vertebrate terminology |
|---|---|---|
| Early erythroblast | Rubriblast | Early rubriblast * |
| Late erythroblast | Prorubricyte | Late rubriblast * |
| Early polychromatic erythrocyte | Rubricyte | Prorubricyte |
| Mid-polychromatic erythrocyte | | Mesorubricyte |
| Late polychromatic erythrocyte | Metarubricyte | Metarubricyte |
| Reticulocyte | Reticulocyte | Reticulocyte |
| Mature erythrocyte | Erythrocyte | Erythrocyte |

* Dr. Erwin Jungherr (personal communication) has offered the helpful suggestion that perhaps the early erythroblast could be called an erythrogonium. If "rubi-" were retained instead of "erythro-," it would be called a rubrigonium. Either of these terms would make the use of early and late unnecessary, because erythroblast or rubriblast would then be limited to mean what is now designated a late erythroblast for avian species.

Rubriblast can be used with or without the modifying term "early" or "late." The photographs by Talbott *et al.*[14] (p. 5, fig. 1, 2) serve as illustrations of early and late stages, respectively, for mammals. In the "Atlas of Avian Hematology," figure 345 is an example of an early erythroblast from avian bone marrow, and figures 346 and 347 are examples of late erythroblasts.[10]

The prorubricyte under the proposed terminology is not a blast cell. It is a stage that has lost its nucleolus, shows condensation of the chromatin to uniformly distributed clumps, and possesses a uniform band of basophilic cytoplasm surrounding the nucleus. Mitochondrial spaces may be variable in amount. The prorubricyte of the proposed classification is the cell shown by Talbott *et al.*,[14] labeled rubricyte.

Mesorubricyte of the proposed classification has a condensed chromatin pattern and may show beginning pyknosis. Pyknosis applies, of course, only to mammals. Other classes of vertebrates do not show nuclear condensation and elimination, and the criterion of the mesorubricyte stage is a neutrophilic reaction of the cytoplasm and further chromatin clumping. During the process of differentiation, ribonucleic acid is being lost and hemoglobin is being accumulated. The first is basophilic and the second is acidophilic. At the midpoint, where both are mixed in equal amounts (based on staining reaction), a gray color is produced.

The cytoplasm of the metarubricyte shows a stronger acidophilic than basophilic reaction. The acidophilia is not as intense as in the mature cell. In mammals it would be equivalent to what Talbott *et al.*[14] have labeled a nucleated red cell (p. 5, fig. 5). In mammals, the nucleus may be present and pyknotic, or may have been lost. In birds and other vertebrates, the nucleus may be round but is usually oval, although not elongated to the same extent as in the mature cell. The chromatin condensation is variable and may be equal to that of the mature cell.

In the A.M.A.[4] terminology, "rubricyte" as a single stage carried no prefix, but there is a distinct advantage in assigning a prefix to each of the three rubricyte stages. The broad term "rubricyte" in the proposed classification has become synonymous with the full range of polychromasia following the blast stage, and "pro-," "meso-," and "mesta-" designate basophilic, neutrophilic, and acidophilic stages, respectively.

There is an advantage in having a prefix for every stage that has the same root name. Then, when the group is to be spoken of collectively, the root without a prefix can be used. Thus, "rubricyte" without a prefix can be used to cover all three stages of the polychromatic series, and is especially useful for study of sectioned

XUM

material where tinctorial gradations are often poor or even lost by the fixation and stain selected.

Many additional special problems arise when specifically naming the stages in the primary erythrocyte series of the embryo in later embryonic generations, and especially under disease conditions. It would seem wise to devote our efforts first toward the establishment of a basic terminology and then later make adaptations and modifications to meet specific special situations.

## THROMBOCYTE SERIES

The thrombocyte or platelet of mammals has its origin in a giant cell with a multilobed nucleus. Its mature stage is a nonnucleated cell fragment. In birds, the thromboblast is very similar to the erythroblast and is never a giant cell. During the process of development, it remains a nucleated cell, and the cytoplasm takes on an oval shape similar to that of the avian erythrocyte. It is obvious that the root "megakaryo-" is a misnomer for the immature stages of thrombocyte development in all classes of vertebrates except the mammals.

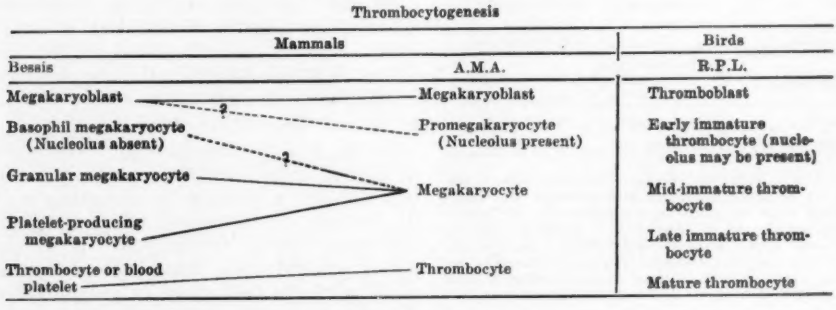
It may be difficult for the mammalian hematologist, who has learned to recognize the various differentiation stages of the megakaryocyte, to realize that, in birds and other classes of vertebrates, the differentiation of the thrombocyte very closely parallels the development of the erythrocyte. The erythroblast and thromboblast are morphologically very similar cells, and the description of one fits the other nearly equally well, except for the presence of hemoglobin in one. The only difference seen between these cells in birds in the early stages of development, before the time of hemoglobin acquisition, is the slightly punctate pattern of chromatin in the thrombocyte nucleus, in contrast to a more delicate reticular pattern in the erythroblast.

Another point that emphasizes the parallelism between erythrocyte and thrombocyte development in birds is the fact that, just as the early embryonic generations of red cells are different from the definitive generations that come later, so are the embryonic thrombocytes different from the definitive cells. A mature erythrocyte is an oval cell, and the mature thrombocyte has a similar shape. In mammals, there is a parallelism in that both erythrocytes and platelets are non-nucleated cells.

Since the A.M.A. terminology deals with definitive stages, the comparisons with avian counterparts will be limited to the same group of stages and will exclude developmental stages found in the chicken embryo.

Finding an acceptable common nomenclature for all classes of vertebrates is not easy. Among mammalian hematologists, differences in description and terminology exist even for the same cell. Some of these problems are illustrated in the following:

Thrombocytogenesis




Descriptions of the cells whereby the various named stages can be recognized are given by Bessis [1] (p. 449), by the A.M.A. (p. 449),[2] and by Lucas and Jamroz (pp. 11–12).[10]

Even in the human field, there is much divergence in descriptions; it is hard to identify homologous terms. In the scheme of names presented for thrombocytogenesis, a vertical line was drawn between birds and mammals. This was intended to indicate

that the named stages of birds can not be homologized with the named stages in mammals at the present time.

Sometime in the future, detailed cytological criteria or cytochemical tests may contribute new information, so that stages that now look so tremendously different can be homologized. For example, it has not been determined that the azurophilic granules of the mammalian platelet are homologous with the specific granules found in the thrombocytes of all vertebrates. The cytoplasmic acidophilia of the immature thrombocytes in cells from man have no counterpart in the chicken. The cells of the latter species sometimes show an acidophilic margin during the degeneration process; otherwise the cytoplasm stains a delicate blue color.

## Lymphocyte Series

Three stages in the development of the lymphocyte are about all that can be distinguished either in the mammalian or avian field. The lymphocytogenesis, the relation of lymphocytes to other cell lines, and the degree of multipotency possessed by lymphocytes have all been difficult to study. Most of the difficulties can be blamed on the cell itself, because it possesses no identifying specific cell structures that serve as firm guides while following the cell through the tissues of the body and and circulating blood.

The brief description presented by the A.M.A.[2,4,5] is fully applicable to birds. It is acceptable because no mention was made of the presence of a nucleolus in the lymphoblast or the prolymphocyte.

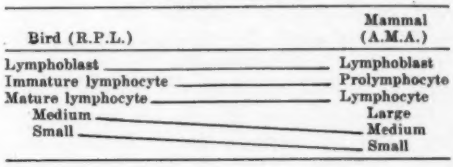
In the descriptions of lymphoblasts in man,[1,6,8,12,14] one or more nucleoli were reported. In impression smears taken from the thymus gland of the chicken embryo,[10] no nucleolus was observed in the lymphoblast, nor even any nonstaining spaces that might have been nucleoli. The cell was large, had a delicate chromatin pattern, and the nucleus was surrounded by a narrow rim of dark, blue-staining cytoplasm with many mitochondrial spaces. It was relatively easy to follow the transition from the lymphoblast to the small lymphocyte in smears from the embryonic thymus gland.

After the bird has hatched, lymphocytogenesis can be followed readily in impression smears from the spleen. The immature lymphocyte identified in the thymus preparations was similar to the prolymphocyte of mammals. The nuclear pattern of this cell was so much like that of the larger lymphocytes in circulating blood that it is questionable whether all lymphocytes found in the circulating blood of the normal chicken are fully mature.

In mammalian hematology, the circulating lymphocytes are sometimes divided into large, medium, and small, or just large and small lymphocytes. In birds, only two categories, medium and small, were established, because it was found that what had often been called a large lymphocyte was, in reality, a blast cell of some particular cell line. It was maintained that it should be designated as an erythroblast, granuloblast, lymphoblast, or whatever happened to be the case. Blast cells are more likely to be found in the circulating blood of normal birds than of mammals.

The following schemes, therefore, seem to be completely interchangeable:

Lymphocytogenesis

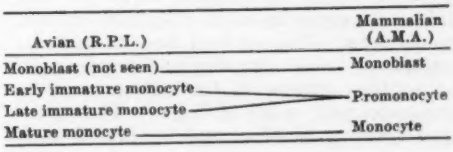



## Monocyte Series

The monocyte series, like the lymphocyte series, can be broken into only about three stages.[2] This is due to the same reason given for the lymphocytes; namely, lack of specific cytological criteria that will allow a clearly recognizable series of distinct stages.

The biggest difference in the immature monocyte of mammals and of birds is the presence of nucleoli in the former.

In birds, three stages of monocyte development have been observed following the monoblast, and the relationship to the A.M.A.[2] series would be as follows:

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The monoblast and promonocyte of mammals have been reported as having a nucleolus by the A.M.A.,[2,4,5] whereas Talbott *et al.*[14] found that nucleoli were no longer visible in the promonocyte stage. Heilmeyer and Begemann [6] described the cytology of the mature monocyte only and considered the existence of a monoblast as a distinct cell questionable. The nucleolus was not mentioned.

Kracke and Garver [8] depicted monocytogenesis from a monoblast, and this in turn from a reticulum cell of spleen and other tissues. Their scheme is similar to the one mentioned earlier, presented by Bessis.[1] Kracke and Garver did not describe either the presence or absence of nucleolus at any stage of monocyte differentiation. All of the monoblasts and promonocytes used for illustration by Osgood and Ashworth [12] were taken from cases of monocytic leukemia. This might have been a factor in the occurrence of the nucleolus in the promonocyte stage of development.

Bessis [1] regarded the monoblast as morphologically identical to the histioblast, and observed that nucleoli were present in the blast stage. He has used the terms "monocyte" and "histiocyte" interchangeably, up to and including the mature form.

In our studies on the chicken, immature stages for the various blood cell lines are often revealed, even in the circulating blood of normal birds. This has allowed us occasionally to observe late immature monocytes in the circulating blood. None of the cells studied were greatly different from the mature cells. The nucleus in the young cell showed a more delicate chromatin pattern than in the mature cell, and the cytoplasm was often lobulated. Nothing was observed to suggest macrophage activity on the part of the cells considered to be late immature monocytes.

Only one early immature monocyte was observed in our studies. It perhaps should have been considered as a monoblast. It did not reveal a nucleolus. The cell was obtained from an impression smear of the spleen, 35 days after the egg had hatched. It was a large cell, with a large nucleus and an extensive layer of cytoplasm around the nucleus. The nucleus had a uniform pattern of small chromatin clumps, similar to the pattern of the typical mature monocyte but more delicate. The cytoplasm was pale blue and contained uniformly distributed blocks of basophilic material. Another cytological feature that helped to identify this cell as a monocyte was the clear, colorless nucleoplasm also characteristic of most mature monocytes, but not found in lymphocytes, either mature or immature.

This cell was not a reticulum cell because, in our material, the reticulum cell, at least as seen in bone marrow, has a conspicuous nucleolus. Neither was this cell a granuloblast, which will be described later. The frequent similarity in appearance of granuloblasts and immature monocytes has been recognized in birds as it has in mammals but, in most immature monocytes observed in birds, the amount of cytosome proportional to the nucleus is greater than for granuloblasts. Also, the chromatin reticulum is more delicate in the granuloblast than in anything we have been able to identify as an early immature monocyte.

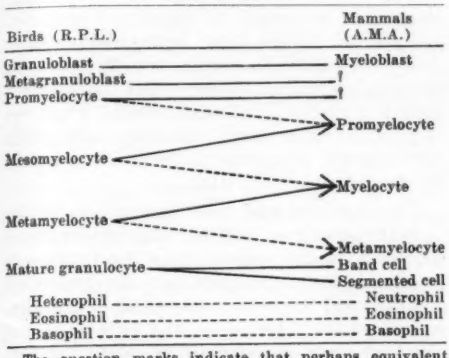
Even typical, normal monocytes are often difficult to identify with certainty in the circulating blood of birds. In the "Atlas of Avian Hematology," ten criteria were established to aid in the separation of monocytes and lymphocytes. One that should be mentioned here because it has proved to be so useful is the occurrence of an azurophilic reticulum of the monocyte cytoplasm. Many, of course, have mentioned azurophilic granules in mammalian monocytes but, to my knowledge, no one has described an azurophilic reticulum superimposed on the basophilic reticulum of the monocyte cytoplasm. When there is an increase in the azurophilic component of the cytoplasm, this material may then take the form of granules. These granules are in turn different from those we have named the magenta bodies of lymphocytes.

### GRANULOCYTE SERIES

Careful consideration was given to the A.M.A. nomenclature when the names were selected to be used in the "Atlas of Avian Hematology," and it was hoped that one set of names would serve both classes of vertebrates. The cytology of the developmental stages in the birds is different from those for mammals, and further research will be needed to fully homologize all the granules and vacuoles observed within the two classes of vertebrates. The following

are the names that have been used for birds and for mammals:

General Terms for the Three Types of Granulocytes




The question marks indicate that perhaps equivalent named stages are absent in the mammals, and the dotted lines with arrows indicate possible homologies if descriptions were changed for both birds and mammals.

Before considering homologies in developmental stages, the name of the adult cell should be discussed first. The term "neutrophil" is applicable only to those granules that are similar to what is observed in the heterophil of man or the frog. The term is not appropriate even for all mammals, since homologous granules of the rabbit and rat take no acidophilic stain. Actually, the neutrophilic granule of man is basophilic and acidophilic, according to Bessis.[1] Keys[9] suggested the term "heterophil" to indicate that, among various species of vertebrates, the specific granule for this cell has a wide variety of forms and staining reactions. This name has been found to be entirely satisfactory for avian terminology and is used for man and mammals by Maximow and Bloom.[11] (*See* p. 55, 7th ed. of their text for colored illustrations of this cell in various species of mammals and the chicken.)

The consideration of the terminology for the developmental stages of the granulocytes will involve examination of name by name and definition by definition.

The granuloblast stage in birds shows two distinct phases—one called a granuloblast and the next, the metagranuloblast. Neither reveals a nucleolus in any smear preparation we have made, and we have examined impression smears from chicken embryos of a wide variety of ages and from various hematopoietic organs. In sections of bone marrow after the egg has hatched, we still found no nucleolus in the granuloblast. It was beginning to appear in the metagranuloblast and was largest and best-developed in the avian promyelocyte, after which it disappeared.

The avian granuloblast, except for the nucleoli, closely resembles the human myeloblast pictured by Talbott *et al.*[14] In the next granuloblast stage, the nucleus becomes eccentric and, in the mass of cytosome opposite the nucleus, many clear vacuoles appear. I have not found any mention in descriptions of human granulopoiesis of the nucleus shifting from a central to an eccentric position in the cell, but any perusal of illustrations will show that such a reaction does occur. However, at the time this is taking place, the azurophilic and specific granules make their appearance, and terminology becomes centered around those cell organella.

The metagranuloblast should be considered a definite stage in heterophil development of birds; its most distinctive feature is the presence of clear vacuoles and its eccentrically placed nucleus. This stage also exists in mammals, but is telescoped with other cellular reactions; vacuolization is not apparent, at least with the usual type of light microscope. This telescoping of the metagranuloblast stage with the promyelocyte stage in birds takes place in the basophil series.

The next stage in the heterophil series is the promyelocyte, and its most distinctive characteristic is the presence of darkly stained magenta bodies that have the same coloration as chromatin material. At the same time, the colorless vacuoles of the metagranuloblast show some pale orange coloration. These, as the intensity of color increases, become the specific granules.

Let us try to pick out homologous entities in blood cell development. Bessis,[1] who examined the cytology of mammalian cells closely, observed that the azurophilic granules of the mammalian promyelocyte sometimes had the shape of rods and commas, and that they stained wine red or bright red. If it could be established that the conspicuous crescents, rings, granules, and solid spheres of birds are homologous with the smaller, paler staining azurophilic granules of mammalian myelocytes, it would go far toward establishing a fixed reference point. The point of con-

XUM

fusion comes when it is stated that azurophilic bodies (mammals) are intermingled with the specific granules, and that the specific granules develop from them.

There has never been any indication that the magenta bodies (birds) produce the specific granules. The definitive rods clearly come from the vacuolar substance of the metagranuloblast, and that is true for heterophils, eosinophils, and basophils. The only parts of the definitive cell that might be derived from some of the small magenta granules are the central bodies of the rods of heterophils.

The difference in nature between the substance of the rod and its central body has been clearly shown recently by Ryerson.[13] It is of interest that Bessis[1] mentioned two components for the specific granule in the human neutrophil—one part basophilic, and one acidophilic. In birds, both parts are acidophilic but differ in solubility.

After discussing the various differences in the cytology of avian and mammalian immature heterophils, how much can we consider as homologous?

The first step is to assume that the azurophil granules (mammals) are equivalent to the magenta bodies (birds). We still have difficulty in homologizing myeloblasts (mammals) and granuloblasts (birds), because azurophilic granules are present in the blast stage of mammals but magenta bodies do not appear until two stages later in birds (except in the development of the avian basophil).

The problem can not be solved by considering the granuloblast and metagranuloblast of birds to be the equivalent of a reticular cell, or a hemohistioblast or a hemocytoblast of mammals; granuloblasts and metagranuloblasts in birds are too definitely identified with the granulocyte line.

The promyelocytes of birds show the precursors of the definitive granules but do not show definitive granules. In the mammalian field, nearly all authors mentioned in the bibliography describe the presence of some definitive, specific granules at the promyelocyte stage.

The second step involves changing the definition of the promyelocyte. If the definition of the promyelocyte for birds was made a little broader, and the definition for mammals was made a little narrower, then the promyelocyte of birds and mammals would be essentially alike. In other words, it would include the transitional stage toward definitive granules, but with only a limited number of definitive granules visible.

This would make it possible for the myelocyte of mammals to equal the mesomyelocyte of birds, and both would show definitive granules from the few of the promyelocyte to 50 per cent of the expected number in a mature cell. Then the metamyelocyte would include cells with the number of granules from 50 per cent to the mature condition.

The nucleus has been ignored in defining these stages, yet for the hematologist working in the mammalian field, nuclear configuration is important. An indented, oval-shaped nucleus is observed in the metamyelocyte, followed by the band cell and then the mature segmented cell.[2] It is a peculiarity of avian blood that the nuclei of immature heterophils do not stain well with the commonly used blood stains, especially Wright's stain. When working with avian blood that has been fixed in an acid and alcohol mixture, a second slide is required to show the nuclear details necessary in making an Arneth or a Schilling count. For studies at this laboratory, we use Petrunkevitch No. 2 as fixative with the May-Grünwald Giemsa stain. Since Arneth counts are time-consuming, they are economically sound only on research projects or in the course of field epidemics where a few birds can be studied intensively to form an estimate of the prognosis for the flock.

In the scheme presented at the first of this section, the solid lines are the homologies as they appear in the present time. If the descriptions of the three myelocyte stages are each drawn about half a stage closer toward the description of the blast stage than they are now, and the ones of the corresponding names in birds are drawn about half a stage toward maturity, then the agreement would be acceptably close, if the first assumption proves to be true.

## PLASMA CELLS

Plasma cells have not been found in the circulating blood of any avian species examined. Mature plasma cells have been seen in considerable numbers in irises of chickens that had been diagnosed grossly as having ocular lymphomatosis. They have been seen in small numbers in the

spleens and in bone marrow of healthy chickens.

Equivalent Terms for Avian and Mammalian Plasmacytogenesis

| Avian (R.P.L.) | Mammalian (A.M.A.) |
|---|---|
| Plasmablast ( not seen) | Plasmablast |
| Early immature plasmacyte | Proplasmacyte |
| Late immature plasmacyte | |
| Plasmacyte | Plasmacyte |

It has been possible to identify an early immature plasma cell from the bone marrow of a healthy bird. The nuclear differentiation was too far advanced to regard such cells as plasmablasts. A plasmablast has not been observed thus far, but its appearance might be anticipated because the early immature plasma cell that was studied had close structural similarity to the primary osteogenic (reticular) cell. The early immature plasmacyte had some similarity to the immature osteoblast of embryonic bone marrow also, but this resemblance perhaps was a superficial one.

There is no serious difficulty in homologizing the stages for birds [10] and mammals.[2]

## CONCLUSION

In conclusion, it is hoped that this discussion of the pros and cons on hematological nomenclature will stimulate a broad approach to a universal and acceptable vertebrate hematological nomenclature, and especially that some hematologists will examine closely and critically the blood cells and the cytology of their development in lower classes of vertebrates. Any final, generally acceptable terminology for the blood cells and their developmental stages will depend on an awareness by the investigator that the full picture of vertebrate hematology is to be found in nothing less than the whole subphylum, and that a single or even several species of one or two classes can not speak for the whole group.

We hear much of rebuilding our stockpile of basic biological information. Comparative hematology is a segment of study that deserves more attention than it is receiving at the present time, and with the multitude of new techniques now available, this should make results and opinions on morphological hematology more conclusive than they have been in the past.

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XUM

# Canine Autogenous Skin Grafting

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THE USE of plastic surgery to correct defects arising from extensive injuries to the integument is common in man. Veterinary literature is meager concerning skin grafting in the dog and cat.

This investigation was undertaken in an attempt to determine the most practical method or methods and the associated techniques involved in successful skin grafting in the dog.

## MATERIALS AND METHODS

Thirty-two dogs were used in these skin grafting experiments. Occasionally, more than one type of graft was performed on a single dog. Observation periods following surgery varied, depending on the success of the graft. Clinical patients were discharged as soon as there was evidence that the graft was successful.

Observations were made with special reference to: (1) percentage of viability of each graft, (2) cosmetic appearance, (3) hair growth, (4) pliability of the graft, and (5) resistance to trauma.

*Preoperative Preparation.*—The areas most likely to require skin grafting, *i.e.*, the legs, were selected as surgical sites. A small number of grafts were also performed on the thorax.

The surgical areas were clipped, shaved, and then scrubbed twice with a liquid soap containing hexachlorophene. The areas were defatted with ether followed by application of 50 per cent isopropyl alcohol. Aseptic surgery was maintained during the entire surgical operation.

Special instruments and equipment consisted of a No. 2 Kalt needle holder, ⅜-circle cutting edge corneal needle, Russian tissue forceps, Von Graefe fixation forceps, 0000 braided black silk, sterile Telfa strips* (an absorbent layer of cotton surrounded by a thin perforated layer of plastic), and Tensor elastic bandages.* A modified Thomas splint was used and the splint incorporated in the bandages.

*Series 1.*—Eleven dogs (3 females and 8 males, 1–10 years old) were used in the first experimental series. A total of 21 free full-thickness skin transplantation experiments were performed.

Areas of skin, 2 by 2 inches, were removed from the ventral lateral wall of the thorax and the lateral aspect of the tibial region. The two segments of skin were then interchanged; the grafts were the same dimensions as the recipient area. As much of the hypoderm as possible was removed to aid revascularization of the transplant. Each corner of the graft was sutured with interrupted sutures (0000 braided black silk). Interrupted sutures were then placed about ¼ inch apart along the graft edges to fix the transplant. Two sterile Telfa strips were placed on the grafts, followed by four or five sterile gauze pads to equalize the pressure. A modified Thomas splint was placed on the leg, with tape applied on the medial aspect of the leg from the tarsal region, over the end of the splint, and up the lateral aspect of the foot to the tibiotarsal region. A 3-inch elastic bandage was wrapped around the sterile sponges and splint. Roller gauze was then applied, followed by 2-inch waterproof tape. The thorax was bandaged in a similar manner, excluding the splint. With the exception of 2 dogs which were used in further experiments, the dogs were observed for a maximum of 25 days and then killed. This afforded sufficient time to develop the surgical procedures and postoperative care, and to observe the grafts.

*Series 2, Delayed and Immediate Transfer of Tube Grafts.*—The results obtained from series 1 experiments were not as satisfactory as anticipated, so it was felt that the use of a delayed transfer of a tube or pedicle graft for use on the extremities of the leg might be more successful in the dog.

This series of experiments was divided into two categories: (1) delayed, and (2) immediate transfer of tube grafts.

Delayed Transfer.—A total of 14 grafts were performed on 8 dogs. One clinical case, a Boxer with a denuded right metatarsal, was used in this series. Since there is little excess skin in the axillary region from which a flap can be removed by undermining and shifting adjacent skin, all surgery was performed on the rear legs.

A pedicle or tube was formed on the medial aspect of the tibial region (fig. 1, 2) by making two parallel longitudinal incisions about 1½ inches apart, leaving the skin attached on both ends. The two parallel incisions were made as far apart as possible, still permitting closure of the area. By grasping the loose skin of the medial region of the tibial area and pulling it upward, it was possible to determine how wide the proposed tube could be cut and still allow for skin closure. The parallel incisions in the skin extended from the femorotibial articulation to within ½ inch of the tibiotarsal articulation. With the aid of a Russian tissue for-

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* Curity (Bauer & Black), Division of the Kendall Co., Chicago, Ill.

ceps and a blunt pointed scissors, the strip of skin was freed from the subcutaneous tissue, leaving the only blood supply to this portion at the attached ends. This strip of skin was formed into a tube by the use of interrupted sutures (0000 braided black silk). The defect from which the flap was raised may be sutured before or after the tube formation, but in this series the tube was formed first. One interrupted stitch was placed near the skin margins at about the center of the skin to be tubed. This suture was left longer so that it could be held by the assistant or grasped with a forceps and used to hold the skin while the remaining interrupted sutures were placed to form the tube. Simple interrupted sutures of medium Vetafil* were used to close the area from which the tube was raised. (It has been found in subsequent work that horizontal mattress stitches are more satisfactory.) When closing this area, two triangles at each pedicle end are formed, one on the tube and the other adjacent to it on the surface from which the tube was raised. When suturing these areas it was important that the sutures should not interfere with the blood supply coming to the tube. A 2-inch stockinet was cut to extend from the hock to the stifle region and taped in place at both ends so that part of the tape adhered to the stockinet and the remainder to the skin. After four days this protective bandage was removed.

* Distributed by Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

After 12 to 40 days the tube plus the desired amount of skin needed to cover the recipient area was reflected distally to cover the lesion on the dorsal aspect of the metatarsal region. It was calculated that the tubed skin would extend to the proximal edge of the skin lesion while the untubed skin extended over the region to be grafted. The sizes of the grafts varied from 1 to 1½ inches wide to 1½ to 3 inches long. Interrupted silk sutures were used. The tension of the skin on the grafted area was that of the normal skin. Tetracycline ointment was applied to the suture line. Two layers of sterile Telfa strips were placed over the graft. Three to four layers of 3- by 3-inch gauze sponges were then applied, followed by a layer of cotton which surrounded the foot from the hock distally. The region of the tube was not bandaged tightly, since this was the only blood supply for the flap. A 2-inch roller-guaze bandage was then applied, followed by adhesive tape. The tips of the toes were allowed to protrude through the bandage. The bandages were changed in three days and then every other day for eight to ten days. The tube furnishing the blood supply to the new graft was ready to be severed after 12 to 14 days.

Immediate Transfer.—Seven tube grafts of immediate transfer were performed, varying in size from ½ to 3 inches wide by 1½ to 3 inches long. Four clinical cases were utilized in this series; 2 of these had severe necrosis of the foreleg following faulty intravenous administration of pento-

Fig. 1—Two parallel incisions made on the medial aspect of the tibial region prior to formation of the tubed graft.

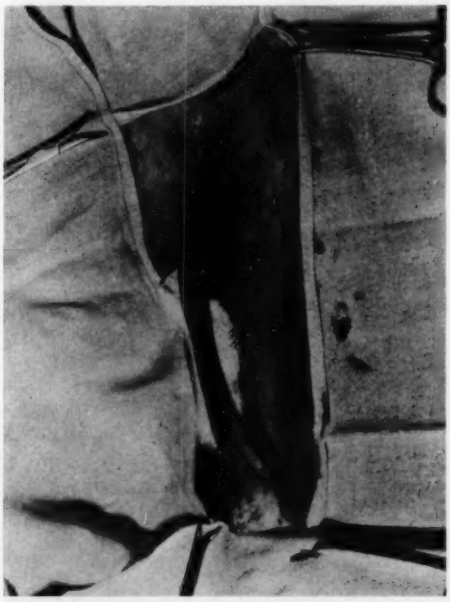

Fig. 2—The tubed flap graft completed by suturing the edges of the elevated strip of skin followed by skin shifting of adjacent skin.

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**TABLE 1—Delayed Transfer of Tube Flap Skin Transplants from the Medial Aspect of the Tibial Region and Thigh to the Dorsal Metatarsal Region**

| Case No. | Width and length of skin for tube formation (in.) | No. of days tube delayed | Size of graft or flap (in.) | Viability of each graft (%) | Length of observation (days) |
|---|---|---|---|---|---|
| 11 | 1½ x 4½ | .... | R*..... | 0 | .....† |
| | 1 x 5 | 25 | L 1¾ x 2 | 100 | 473 |
| 101 | 1¼ x 5 | 20 | L 1¼ x 1½ | 100 | 526 |
| 102 | 1¼ x 5 | 32 | L 1½ x 2 | 100 | 491 |
| | 1 x 3¾ | 30 | R 1 x 1½ | 100 | 362 |
| 104 | 1¼ x 5 | 20 | L 1¼ x 2 | 100 | 527 |
| | 1¼ x 5 | 20 | R 1¾ x 1¾ | 100 | 527 |
| 105 | 1¼ x 3¼ | 39 | L 1½ x 2½ | 100 | 216 |
| | 1¼ x 3¼ | 39 | R 1½ x 2½ | 100 | 216 |
| 106 | 1¾ x 5 | 24 | L 1½ x 2¾ | 100 | 123 |
| | 1¾ x 5 | 24 | R 1½ x 2½ | 100 | 123 |
| 107 | 1 x 3¾ | 40 | L 1 x 1¼ | 100 | 186 |
| | 1 x 3¾ | 40 | R 1 x 1¼ | 100 | 186 |
| 14404** | 1½ x 3 | 12 | R 1½ x 3 | 85 | 14 |

* R = Right rear leg; L = left rear leg. † Tube destroyed on day 6. ** Clinical case.

barbital sodium. The third dog had sustained a severe pressure necrosis of the flexor surface of the right metatarsal region due to faulty bandaging, and the fourth had a chronic ulcerative dermatitis of the dorsal aspect of the left metatarsal region. The remaining three grafts were conducted on experimental dogs. Two grafts were transferred to the dorsal metatarsal area, utilizing the skin of the medial aspect of the tibial region and thigh. The third graft was removed from the anterolateral aspect of the humeral area to cover a lesion of the dorsal radio-ulnar region.

**Fig. 3—The completion of an immediate transfer of a tubed flap graft on the dorsal metatarsal region.**

The technique for the formation of the tube for immediate transfer was the same as that for the delayed transfer, except that after the tube was formed the transplantation was made immediately rather than after a delay of several days to weeks. A tubed flap graft which was transferred immediately after formation is shown (fig. 3). Suturing and bandaging of the grafts were the same as for the delayed transfer of pedicle grafts. The grafts were observed for 15 to 523 days.

*Series 3, Free Full-Thickness Grafts.*—A total of 31 free full-thickness grafts were made, ½ to 3 inches wide by 1 to 3½ inches long. Five were used on granulating wounds; 26 were placed on fresh wounds (tables 3, 4).

In 3 clinical patients six grafts were performed; two of these grafts were applied to granulating lesions. Dog 301 was a black Labrador Retriever with a chronic ulcerative granulomatous lesion initially caused by a wire cut on the left metatarsal region and followed by subsequent licking of the area. The other granulating lesion was in a Pomeranian (19350) which had been hit by a car, resulting in a denuded area on the dorsum of the carpus to the extremities of the digits. Dog 18337 was a 2-year-old Dachshund with third-degree burns of the face resulting in tissue formation and disfiguration of the upper eyelids. Four grafts were made to cover this area.

Of the 25 experimentally produced lesions, three were granulomatous. The donor site for 14 of the grafts was the medical aspect of the thigh; the recipient area was the dorsal aspect of the left and right forelegs. Six grafts were transferred from the thorax to the tibial region; one of these grafts was applied to a granulating lesion. Other grafts were transferred from the thorax, medial thigh, or lumbar areas to the dorsal metatarsal area or from the thorax to the forelegs (tables 3, 4).

Preoperative preparation and draping was similar to that described in series 1. The size of the skin to be removed for the graft was predetermined by using a sterile gauze sponge or a sheet of sterile aluminum foil cut to the desired size. An

antibiotic ointment such as tetracycline hydrochloride was applied along the edges of the skin. It was difficult to prevent contamination in the region of the extremities, especially when it was necessary to observe the results at relatively short intervals for this study. Two sterile Telfa strips were placed on the graft followed by sterile gauze sponges. One of the sponges was unfolded to encircle the other bandages and thus assist in keeping them fixed in position. A thin layer of absorbent cotton was then placed around the dressings extending from the elbow joint distally to the paw, leaving the tips of the digits exposed. Two-inch roller gauze was snugly wrapped around the cotton. Waterproof adhesive tape was then spiraled around the leg, starting proximal to the tip of the toes and continuing to the next joint. For the six grafts from the thorax to lateral tibial region, bandaging was the same as for series 1. The four grafts of the head region were bandaged in a somewhat similar fashion to those of the legs. Bandages were changed every two days unless loosened by the dog prior to that time. The grafts were observed from 14 to 382 days.

**TABLE 2—Immediate Transfer of Tube Skin Transplants**

| Case No. | Donor and recipient area | Size of graft or flap (in.) | Viability of each graft (%) | Length of observation (days) |
|---|---|---|---|---|
| 102 | Medial left tibial region to dorsal metatarsal area | 1¼ x 1¾ | 0 | 523* |
| 107 | Anterio-lateral-humeral region to dorsal radio-ulnar area | 1½ x 2 | 75 | 171 |
| 201† | Right axillary region to dorsal radio-ulnar area | 1 x 3 | 85 | 80 |
| 202 | Medial right tibial region to dorsal metatarsal area | 1¼ x 1¼ | 90 | 30 |
| 08010† | Left thorax to dorsal left metatarsal area | 2 x 3 | 60 | 14 |
| 08561† | Medial right tibial area to flexor aspect of hock | 1½ x 2 | 75 | 19 |
| 47261† | Right axillary region to dorsal radio-ulnar area | ¾ x 1½ | 85 | 17 |

* Dog was used for other series. † Clinical cases.

*Series 4, Split-Thickness Grafts.*—Five split-thickness grafts were made on experimentally produced nongranulating lesions.

A Brown dermatome* was used to remove these grafts. All transplants were cut at a thickness of 0.030 inch, with the exception of one which was removed at 0.035 inch. An assistant tensed the skin anterior to the donor area while the operator

* Zimmer Manufacturing Company, Warsaw, Ind.

**TABLE 3—Free Full-Thickness Grafts Applied to Nongranulating Wounds**

| Case No. | Donor and recipient area | Size of graft and area grafted (in.) | Viability of each graft (%) | Length of observation (days) |
|---|---|---|---|---|
| 11 | Left thorax to left tibial region* | Thorax 1¼ x 1¼ | 60 | 319 |
| | | Tibial 1¼ x 1¼ | 0 | 7 |
| 11 | Right thorax to right tibial area* | Thorax 2 x 2 | 50 | 303 |
| | | Tibial 2 x 2 | 0 | 6 |
| 11 | Left lumbar area to dorsal left metatarsal area | 1¾ x 1½ | 90 | 441 |
| 11 | Medial thigh area to right and left forelegs | L 1¾ x 1½† | 10 | 199 |
| | | R 2 x 1½ | 0 | 7 |
| 101 | Right thorax to right tibial area | 2¾ x 2¾ | 60 | 716 |
| 101 | Medial thigh region to right and left forelegs | L 1¾ x 1¼ | 100 | 321 |
| | | R 1¾ x 1¼ | 0 | 6 |
| 102 | Medial thigh region to right and left forelegs | L 1½ x 1⅛ | 10 | 65 |
| | | R 1½ x 1¼ | 75 | 65 |
| 104 | Medial thigh region to right and left forelegs | L 2 x 1½ | 100 | 263 |
| | | R 1¾ x 1¼ | 100 | 263 |
| 105 | Medial thigh region to right and left forelegs | L 2¾ x 1½ | 0 | 6 |
| | | R 2 x 1¾ | 0 | 6 |
| 107 | Left thorax to right and left forelegs | L 1½ x 1 | 0 | 6 |
| | | R 1½ x 1 | 80 | 32 |
| 208 | Medial thigh region to right and left forelegs | L 1¾ x 1 | 20 | 20 |
| | | R 2 x 1½ | 0 | 9 |
| 209 | Medial thigh region to right and left forelegs | L 2 x 1 | 100 | 17 |
| | | R 2 x 1½ | 50 | 17 |
| 18337** | Right thorax to over right eye | 1¼ x ¾ | 100 | 193 |
| 18337** | Left thorax to over left eye | ½ x ¾ | 100 | 170 |
| 18337** | Left thorax to center of forehead | 1 x 1 | 95 | 170 |
| 18337** | Right thorax to forehead area | 1 x 1 | 100 | 77 |

* Grafts were interchanged. † R = right foreleg, L = left foreleg. ** Clinical case.

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placed tension in an opposing direction with one hand and with the other manipulated the dermatome. The size of the transplants ranged from 3 to 4 square inches. After removal, the remaining dermal layer was removed as evenly as possible with a sharp scalpel so that this area could then be used for the graft bed. When this was completed and hemorrhage controlled by sterile pressure packs, the split-thickness graft was then replaced in the area from which it had been removed.

Continuous sutures (0000 braided black silk) were used to fix the transplant to the wound edges. Tetracycline hydrochloride ointment was applied over the graft. Two layers of Telfa strips were applied, followed by three to four layers of sterile 3- by 3-inch sponges which were unfolded prior to placing on the area. Interrupted sutures of medium Vetafil were placed in each corner of the sponges so that they included all of the dressings and a

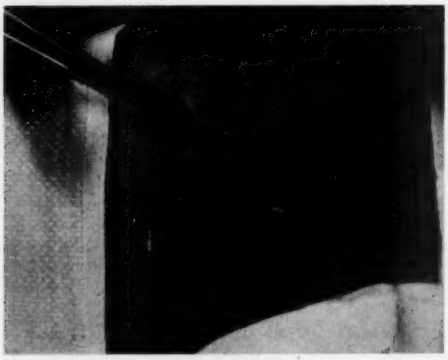

**Fig. 4—Excision of the bleb of skin from the donor area to complete the removal of the small deep graft.**

small amount of skin surrounding the margin of the graft. One or two extra stitches were placed on the longer sides of the sponges. One roll of 3-inch elastic bandage was then applied over the dressings and continued so as to encircle the thorax. Three-inch roller gauze was placed over this, followed by 2-inch waterproof adhesive tape applied so it incorporated part of the skin and hair at both margins of the bandages. The bandages were removed in three to five days and redressed. A bland ointment was applied to the area for one to two weeks to keep the skin pliable. The grafts were observed for 84 to 350 days.

*Series 5, Small Deep Grafts.*—The technique for obtaining the small deep grafts in this series was similar to that described by Davis.[6] Five dogs were used to produce eight graft areas.

The medial aspect of the thigh was selected as the donor area. Lesions were experimentally produced on the thorax. The size of the lesions ranged from 1¾ to 5 inches wide by 2 to 5 inches long. They were allowed to granulate seven to 16 days until a suitable "seed bed" was obtained. The area was considered acceptable for grafting when the granulation tissue was firm and bright red, indicating that a good blood supply had been established.

Prior to actual seeding of the area, the lesion was cleansed with gauze sponges saturated with ether as suggested by Davis.[5] Sterile sponges saturated in 1:3,000 solution of aqueous benzalkonium* were then allowed to remain on the area for at least 15 minutes. The small deep grafts were obtained by placing a straight atraumatic intestinal needle in the jaws of a Mayo needle holder so that ½ to ¾ inch of the needle protruded beyond the distal end. The tip of the needle was inserted into the dermal layer of skin on the medial aspect of the thigh, the donor area. A small bleb of skin was elevated and removed with a sharp scalpel (fig. 4) cutting with a slant downward to the center and then slanting the scalpel upward so as to have full thickness only at the center. These grafts were then placed on the dry recipient area ⅜ inch apart. The grafts were oval in shape and ranged in size from 2 to 4 mm.

Furacin-penicillin ointment† was placed between the seeds prior to the application of the dressings. The bandaging procedure was similar to that of series 4. Five days postoperatively the dressings were removed and the graft area checked. New dressings were applied and left in place for three to five days longer, depending on the condition of the transplants at the time of the first examination. Bandages were left in place for at least ten to 14 days, or a few extra days if the dog persisted in licking the area.

*Series 6, Histological Study.*—The last series was completed in an effort to study the macroscopic and histological changes. At 2-day intervals a portion of each graft was removed for histological study until the last dog in the series had yielded a 22- and a 24-day-old graft.

## RESULTS

Results obtained from the skin transplantations in each series were recorded; observations were made on the following factors: (1) percentage of viability of each graft, (2) cosmetic appearance of the grafts, (3) hair growth, (4) pliability of the graft, (5) resistance to trauma, and (6) histological and pathological changes of the graft following transplantation (series 6 only).

*Series 1.*—The viability of grafts was determined solely on the basis of macroscopic appearance. Only areas which indicated satisfactory vascularization, as evidenced by color changes, were considered as "takes." For the first 48 postoperative hours the transplants were black and blue

* Aqueous Zephiran, produced by Winthrop Laboratories, Inc., New York, N.Y.

† Eaton Laboratories, Inc., Norwich, N.Y.

Fig. 5—Two tubed flap grafts 135 days following transplantation on the dorsal metatarsal region.

with little hint of a blood supply. The grafts gradually lost the darker colors and, after 72 to 96 hours, began showing a light red tinge which became more pronounced each day. Usually by the seventh to the eighth day a light red color had permeated the graft. In this series, viability was poor due to loosening of bandages and lack of patient restraint. Probably the failure to remove all of the subcutaneous tissue from the graft before placing it on the donor area was also a factor.

Only 2 of the 11 dogs used in this series were kept a sufficient time to make any evaluations concerning cosmetic appearance, hair growth, pliability of the skin, and resistance to trauma. Dog 3 was observed for 162 days. This graft was a complete "take." Hair growth was sparse, but the graft was pliable and was resistant to trauma. There was some pigment change; the graft was darker than the adjacent skin. Dog 11 was observed for 671 days. Only 60 per cent of the graft survived, but appeared to have normal color and resisted trauma well. Hair growth was reduced by at least one half.

*Series 2.*—Delayed Transfer of Tube Grafting.—In the delayed tube transplants (table 1), the period before the tubes were shifted to the new graft beds was 12 to 40 days. The more adequate the circulation in the tube prior to completion of the transplant, the less change in color evidenced by the graft following transplantation. Those pedicles which were delayed 30 to 40 days showed a red tinge in two days.

The cosmetic appearance of the grafts was good, except for the demarcation at the border and the difference in density and direction of hair growth. The appearance of a graft 135 days after transplantation is shown (fig. 5). A graft of 96 days with the hair growing in the wrong direction is also shown (fig. 6). The hair was longer and less dense because the transplant had its origin from the medial aspect of the thigh. The pedicle in this case was not severed so that it could be observed whether there would be any change in the size of the tube after the flap had developed a blood supply of its own. There appeared to be a decrease of about one third in the tube diameter.

The pliability of the grafts was evaluated by palpation of the graft and the adjacent skin. After three weeks there was little or no difference.

Resistance to trauma was observed under kennel conditions, and this could be misleading. However, when the dogs were inactive there was a greater opportunity for the dogs to molest the grafts just from boredom. One dog severed the tube shortly after it was formed.

Immediate Transfer of Tube Grafts.—The color of the grafts which were trans-

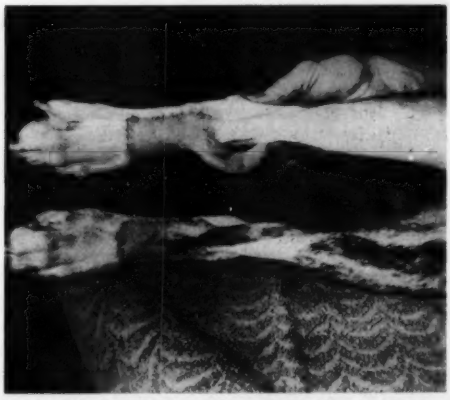

Fig. 6—Two tubed grafts 94 days after transplantation. The pedicles were not servered. Notice the direction of the hair growth.

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ferred immediately seemed to indicate that the blood supply was not as adequate as that of the delayed tube flap grafts. All of the grafts progressed through color changes similar to those of free full-thickness grafts in series 1. The periphery of the grafts seemed to show a greater lack of blood supply than did the center and the region nearest the tube. No 100 per cent "takes" were achieved (table 2). Even though the length of the tube was relatively short, there evidently was not a satisfactory blood supply through the pedicle to nourish the flap until it developed a blood supply of its own.

The cosmetic appearace of the grafts was satisfactory, although the areas which failed to remain viable detracted from the over-all appearance. The rate of hair growth of the grafts was not changed significantly following the transplantation, and pliability of the skin was satisfactory, resembling that described in the preceding section. The grafts were not affected by trauma during the observation period.

*Series 3.—Grafts Applied to Nongranulating Lesions.*—The 26 grafts applied to nongranulating lesions appeared to follow a characteristic pattern of vascularization. On the second day there was only a slight indication of the development of a new blood supply. The grafts had a bluish tinge with a faint reddish color. By the third and fourth days the reddish tinge became more evident. At the end of six days the successful grafts were a vivid red. The critical period for the transplants was the first ten days; complete losses occurred during this period (table 3). The most serious cause for loss of the grafts was a loosening of the bandages during the crucial period.

The cosmetic appearance of the transplants can not be evaluated separately because it was affected by the hair growth and the pliability of the graft. The density of the hair on the graft was dependent somewhat on the extent to which the hypodermis was removed from the donor skin prior to transplantation. It was observed in this series that there was a decrease in hair growth in a small percentage of the cases. In dog 101, the graft was transferred from the right thorax to the right tibial region. This was the only graft of the series in which a pigment change was noted.

Resistance to trauma appeared to be satisfactory following the first 21 days. However, dog 104 was noticed licking the graft 200 days following transplantation. A mild erythema resulted but subsided in three days with no treatment. There was no recurrence of this condition during the remainder of the observation period.

*Grafts Applied to Granulating Lesions.*—Five grafts were completed on granulating lesions (table 4).

In clinical patient 301, a dog with an ulcerative lesion of the dorsal aspect of the left metatarsal region, all of the graft became necrotic after ten days of questionable viability. Experimental dog 101 followed a similar pattern. Clinical patient 19350 had a lesion of the dorsal carpal-metacarpal area following an automobile accident which denuded an area ¾-inch wide by 2¾-inches long. After 13 days, satisfactory granulation had occurred so that a graft was attempted. This transplant required five days before a satisfactory reddish tinge appeared and 12 days before it could be ascertained if the tissue would be viable. A small erosion of the epithelium occurred in the center of the graft on the twelfth day. The grafts of dog 102 followed a similar pattern. There appeared to be a delay in vascularization of the grafts with a lower percentage of "takes" than was evident for those transplants applied to nongranulating lesions.

Although there were only five grafts to evaluate, the cosmetic appearance, hair

**TABLE 4—Free Full-Thickness Grafts Applied to Granulating Wounds**

| Case No. | Donor and recipient area | Size of graft (in.) | Viability of each graft (%) | Length of observation (days) |
|---|---|---|---|---|
| 101 | Medial right thigh region to right dorsal metatarsal area | 1½ x 1 | 10 | 382 |
| | Right thorax to right dorsal metatarsal area | 2 x 2 | 0 | 25 |
| 102 | Left thorax to left tibial region | 2 x 2 | 60 | 567 |
| 301* | Left thorax to left dorsal metatarsal area | 3½ x 3 | 0 | 14 |
| 19350* | Right thorax to right carpal region | 2½ x ½ | 80 | 14 |

* Clinical cases.

growth, pliability, and resistance to trauma appeared to be similar to those transplants applied to nongranulating wounds.

*Series 4.*—Some of the more pertinent data associated with the split-thickness grafts are given (table 5). On the fourth day the grafts appeared to be somewhat blue, interspersed with tinges of red. By seven days, the grafts were red. After 14 days, the bandages were not replaced.

The grafts were not traumatized by the dogs under kennel conditions. The fact that the transplants were located on the lumbar and dorsolateral thoracic wall tended to lessen the chances of injury.

*Series 5.*—There was a decrease in size of the original wound in contrast to the size of the same lesion following the period allowed for a satisfactory granulation bed to occur (table 6). The viability of the seed

**TABLE 5—Split-Thickness Skin Grafts Removed with a Dermatome**

| Case No. | Donor and recipient area | Size of graft (in.) | Viability of each graft (%) | Length of observation (days) |
|---|---|---|---|---|
| 11 | Left thorax to right thorax | 4 x 4 | 40 | 350 |
| 101 | Right thorax and replaced to same area | 4½ x 4½ | 95 | 337 |
| 102 | Left thorax to right lumbar area | 2 x 2 | 50 | 334 |
| | Left thorax to right thorax | 3¼ x 3 | 90 | 200 |
| 105 | Right thorax and replaced to same area | 4 x 3½ | 75 | 84 |

Cosmetically, the grafts were not as satisfactory as those resulting from the tube flap or the free full-thickness methods. The transplants developed a scaly appearance about the eleventh day. This tended to persist indefinitely, although it was not as severe as during the first few weeks. Dog 101 was observed for 337 days, during which time there was a marked exfoliation from the transplanted area. This graft was cut at a depth of 0.035 inch, which probably is very near the complete depth of the normal skin in that area. When examined microscopically, there was evidence that sebaceous and sudoriferous glands were present, indicating that the graft might have included all of the skin layers. In spite of this there was some shrinkage and considerable scaling of the transplant. All of the other grafts were cut at 0.030 inch.

Hair growth was not observed on the grafts except in dog 101, in which the graft might have been nearly a full-thickness transplant. A small amount of hair had grown from the graft after 42 days (fig. 7). All of the other grafts were hairless because the hair follicles are located in the deeper layers of the corium and the hypodermis.

The pliability of the transplants left much to be desired. Even after several months the grafts had a "leathery" character when palpated. Very little subcutaneous fat accumulated underneath the split-thickness grafts, which made them less pliable than the full-thickness grafts.

grafts was determined by macroscopic examination. Only the grafts which were present at the end of the observation period were counted as being viable.

The cosmetic appearance of the transplants was not as satisfactory as those of the free full-thickness or pedicle grafts. However, they were more satisfactory than the split-thickness grafts. A "cobblestone" effect resulted on the healed area, both at the donor and recipient site. Hair growth was very scanty in this type of graft, since only a few hair shafts appeared on each island of skin.

The pliability of the grafted area did not approach that of a free full-thickness nor a pedicle transplant. The skin felt somewhat roughened and very little subcutaneous tissue was present to add to the pliability of the area.

Resistance to trauma was somewhat better than in split-thickness grafts. In areas where the seeds were too far apart to cause a coalescing of the adjacent islands of skin, scar tissue epithelium covered the area, leaving it thin and susceptible to trauma.

*Series 6.*—From the limited histopathological studies made, there appeared to be degenerative changes in the transplant for the first eight to ten days, at which time a balance was reached and regeneration of the surviving elements exceeded degeneration. The epidermis appeared to show the severest degenerative changes, although there were also changes in the dermis involving some of the blood vessels. The hair

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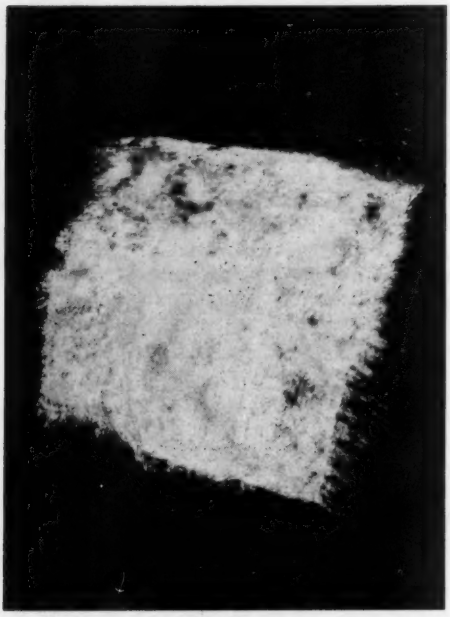

**Fig. 7—A split-thickness graft in the thoracolumbar region 0.035 inches thick, 42 days after transplantation.**

follicles and sebaceous glands showed the least degenerative changes. Microscopic and macroscopic examination of the grafts studied seemed to indicate that the blood supply was adequate in 12 days. The transplants were returning to a normal status in 24 days, which was the termination of this study.

## DISCUSSION

The basic surgical techniques which are successful for skin transplantation in the human species are also applicable to the dog. However, bandaging and postoperative care differs considerably. Restraint, effective bandaging, and the possibility of contamination are problems which become magnified in the dog.

Four types of skin grafts were attempted on experimental animals and clinical cases. It was found that there was no single type of graft which would meet all of the requirements of a successful graft on all regions of the body.

It was concluded that the delayed tube flap or pedicle graft was the most successful with regard to percentage of viability. This method was well adapted to lesions on the extremities of the rear legs. The formation of a delayed tube graft on the forelegs would have been more difficult due to a lack of sufficient skin in the axillary region. Although this technique gave the best results, it appeared to be best adapted to the rear legs, since the tube could be formed on the medial aspect of the tibial region and thigh, thus lessening the chances of a conspicuous scar. This graft is limited to relatively small areas of transplantation because the area of skin removed for the tube must either be covered with another graft or by "skin shifting." If the tube is excessively long there is the possibility of a faulty blood supply to the tube with necrosis of the pedicle.

The immediate tube flaps were not as successful as the delayed pedicle, although

**TABLE 6—Small Deep Grafts Performed on Granulating Lesions**

| Case No. | Original size and location of lesion (in.) | Size of lesion (in.) following specified granulation period | No. grafts "seeded" | No. grafts survived | Observation period (days) |
|---|---|---|---|---|---|
| 301 | 3 x 1¾ right thorax | 2 x 1 10 days | 11 | 5 | 40 |
| 302 | 5 x 5 left thorax | 2 x 3½ 9 days | 30 | 10 | 33 |
| | 3 x 3⅝ right thorax | 2¾ x 1¾ 8 days | 22 | 21 | 12 |
| 303 | 2 x 2¼ left thorax | 1¼ x 1 16 days | 10 | 2 | 60 |
| | 3½ x 3¼ right thorax | 2¼ x 2½ 7 days | 17 | 11 | 27 |
| 304 | 3½ x 4½ right thorax | 2⅞ x 2⅜ 11 days | 11 | 0 | 56 |
| | 4 x 3 left thorax | 3 x 2 7 days | 21 | 8 | 34 |
| 305 | 2¾ x 2¼ right thorax | 2 x 1¾ 7 days | 43 | 27 | 53 |

they gave promise of being valuable in transplants on the extremities. Three of these grafts, used on the forelegs, were partially successful. The remaining grafts were placed on the metatarsal and tibiotarsal region. It was difficult to immobilize the leg and maintain sufficient pressure on the transplant without interfering with the blood supply of the tube.

Thirty-one free full-thickness grafts were made on granulating and nongranulating lesions. This type of graft is the most difficult to accomplish. The clinical application for this type of graft includes covering areas of the legs or face following surgical removal of large tumors in which the denuded areas can not be covered by skin shifting. The cosmetic appearance, hair growth, and pliability of the skin is excellent, if a satisfactory "take" can be accomplished.

The small deep grafts or seed grafts are not as cosmetically acceptable as either the free full-thickness or the pedicle grafts. Since the epithelization has to occur from the periphery of each seed, the grafts must be sufficiently close together so that they eventually coalesce. It is doubtful whether an area less than 3 inches square should be grafted due to the looseness of the skin on the thorax and the resultant shrinking of the wound. The area was not as resistant to trauma as the pedicle or free full-thickness transplants. Since the seeds were not originally attached to the seed bed, trouble was experienced in immobilizing the grafts. This was corrected by suturing the dressings to the adjacent skin.

The split-thickness graft, although one of the easiest to obtain a "take," had many objectionable features. Difficulty was experienced in finding a suitable donor area for the use of the dermatome, since the dog has very few flat areas of skin. The shrinking and scaling of the transplants were objectionable. Also, there was no hair growth. Since the grafts were not as resistant to trauma as the full-thickness transplants, it would not be advisable to use this type of graft on the extremities of the dog where it would be more susceptible to injury. One of the main indications in dogs would be following severe burns where extensive areas of the integument had been destroyed.

Injuries to the grafts from trauma following successful transplantation were not a problem in this study. Most of the dogs were kept in kennels; it is possible that a dog which is free to run might subject the graft to injuries which would not occur under kennel conditions.

There was no series in this study related to the returning of innervation to the various types of grafts. This would be most interesting if a satisfactory means of evaluating the sensations of pain, cold, and heat could be devised for the dog. This information would be valuable since the transplant is in danger of severe trauma until such time as the area becomes reinnervated.

## Summary and Conclusions

1) Four different types of autogenous skin grafting techniques for use on the dog involved a total of 98 grafts.

2) Restraint, maintenance of a satisfactory pressure bandage, and prevention of contamination of the grafted area appeared to be the greatest difficulties encountered in this study. With proper observance of these prerequisities, a satisfactory percentage of grafts can be successful.

3) No single type of skin graft was uniformly successful for all areas of the dog's body nor for all kinds of skin lesions.

4) The delayed transfer of a pedicle graft appeared to be the most successful and satisfactory transplant for lesions of the extremities, especially the rear legs.

5) The free full-thickness graft was relatively successful for lesions free from infection and excessive granulation tissue.

6) The small deep graft, although easy to perform, does not result in as good a cosmetic appearance as either the free full-thickness or the pedicle transplant since it results in a "cobblestone" or "pitting" effect at both donor and recipient areas.

7) A satisfactory split-thickness graft requires expensive equipment primarily designed for use of the human species. It is cosmetically objectionable since there is no hair growth and scaling of the epithelium persists for several weeks or months. The resistance to trauma is not as good as for the full-thickness or the pedicle graft, so it should be used only over the lumbar or dorsal thoracic region where it is less likely to be injured.

8) Areas on the thorax less than 3 by 3 inches need not be grafted, as cicatrization tissue will result in a complete closure of the lesion in three to five weeks with very little scarring due to the looseness of the integument in that area.

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9) From the limited histopathological work done on the transplants it appeared that there were considerable degenerative changes involving the epidermis and the upper layers of the dermis during the first eight to ten days, at which time the regenerative processes equalized the degenerative changes. The blood supply appeared adequate by the twelfth day and the transplants were rapidly returning to a nearly normal status by 24 days, which was the termination of the study.

10) Skin grafting in the dog has its greatest indications in facial lesions and injuries to the skin of the extremities.

11) Injury to the grafts as a result of trauma following successful transplantation were negligible in this study.

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# The Narcotic Analgesics and Their Antagonists as Adjuncts to Barbiturate Anesthesia. I. Physiological and Pharmacological Considerations

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THE PURPOSE of this communication is to present certain aspects of the pharmacology of the narcotic analgesics and their antagonists, and to discuss their use in conjunction with barbiturate anesthesia. The chemical structures of the drugs to be discussed is shown (graph 1). Levorphanol tartrate* does not have the oxygen bridge between carbons 4 and 5 of the morphine nucleus, and the 7-8 linkage in ring 2 is saturated. Pharmacologically, it has about twice the potency of morphine in all parameters of activity.[2] Nalorphine † and levallorphan** differ from the parent compounds by the substitution of an allyl group for the methyl radical on the tertiary nitrogen (position 17). Meperidine‡ and alphaprodine§ are similar pharmacologically, although some interesting differences do exist.

## MATERIALS AND METHODS

A total of more than 100 experiments were performed on 50 dogs. Twenty unanesthetized dogs were used to show the effects of the antagonists on sedation induced by the narcotic analgesics. The rest of the experiments were performed on dogs anesthetized with pentobarbital sodium and subsequently given the narcotic analgesics. Oxygen consumption and respiratory patterns were recorded by spirometry utilizing the Benedict-Roth metabolism apparatus. Oxygen (100 %) was inspired by the dogs throughout each experiment. Blood gas determinations were performed according to the manometric method of Van Slyke and Neill.[23] Cardiac output was determined by the direct Fick principle employing right heart catherization through the superficial neck veins.[4] Simultaneous recordings of the electrocardiogram, femoral arterial blood pressure, and respiration were made with an ink-writing polygraph* equipped with strain gauge transducers† for recording blood pressure and pressure transducers * for recording respirations.

## RESULTS

Ten dogs were deeply sedated by the hypodermic administration of morphine (2.0 mg. per pound of body weight or 4.4 mg./kg.). Forty-five to 60 minutes after the morphine, each dog was given levallorphan (0.10 mg./lb.) or nalorphine (0.5 mg./lb.) intravenously. Initial effects of the antagonists were observed within 15 second after injection. A deep inspiration was followed by the conversion of the panting type of respiration, characteristic of the influence of morphine,[4] to breathing of a greater tidal volume and somewhat slower rate. Ninety seconds after the antagonist was administered, almost all the obvious signs indicative of morphine hypnosis had been overcome. The dogs were alert and responded rapidly to all the usual stimuli. A mild degree of sedation persisted for one to two hours, but the dogs remained responsive and were aroused easily by slight stimuli.

Eight dogs were sedated by the intramuscular administration of meperidine (10.0 mg./lb. or 22.0 mg./kg.). Forty-five to 60 minutes after meperidine administration, each dog was given levallorphan (0.1 mg./lb.) or nalorphine (0.5 mg./lb.) intravenously. The recovery from meperidine sedation was similar to the recovery from morphine except that respiration, less prominently depressed by meperidine, did not change as markedly following the administration of the antagonist.

Dogs were anesthetized to plane 1 or plane 2 of surgical anesthesia. A cuffed endotracheal tube was passed into the tra-

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* Levorphanol tartrate (Levo-Dromoran), ** levallorphan tartrate (Lorfan), and § alphaprodine hydrochloride (Nisentil) are produced by Hoffmann-LaRoche, Inc., Nutley, N.J.; † nalorphine hydrochloride (Nalline) by Merck, Sharp and Dohme, division of Merck & Co., Inc., Rahway, N.J.; and ‡ meperidine hydrochloride (Demerol) by Winthrop Laboratories, Inc., New York, N.Y.

* Manufactured by the Grass Instrument Company, Quincy, Mass.

† Manufactured by Statham Laboratories, Los Angeles, Calif.



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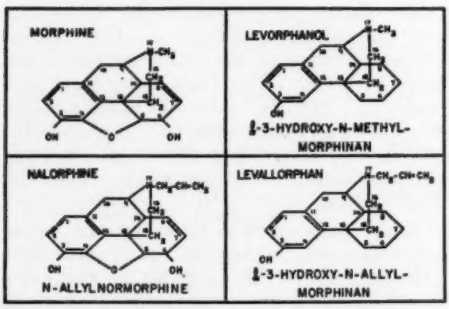
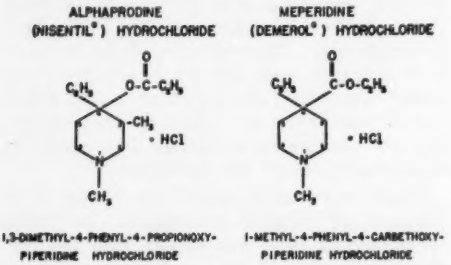
**TABLE 1—Effect of Morphine Administered Intravenously to an Anesthetized Dog, Followed by Levallorphan Administered Intravenously**

| Items * | Pentobarbital alone | Twenty minutes after morphine | Av. of six minutes after levallorphan |
|---|---|---|---|
| Respiratory rate | 7.90 | 6.00 | 11.30 |
| Tidal volume (cc.) | 152.00 | 162.00 | 202.00 |
| Minute volume (cc.) | 1,141.00 | 770.00 | 2,065.00 |
| Oxygen consumption (cc./min.) | 78.00 | 76.90 | 78.90 |
| Ventilatory equivalent | 0.0684 | 0.0999 | 0.0382 |
| Arterial $CO_2$ (volumes %) | 51.90 | 56.80 | 52.00 |
| Arterial $O_2$ (volumes %) | 20.85 | 21.10 | 21.20 |
| Arterial-venous oxygen difference (volumes %) | 3.90 | 3.10 | 1.80 |
| Cardiac output (liters/min.) | 2.20 | 3.40 | 4.30 |

* Averages of 4 dogs.

chea and connected to the spirometer. Electrocardiogram, blood pressure, and respiration were monitored on the polygraph. Blood samples were obtained from the opposite femoral artery and from the right heart. Morphine (1.5 mg./lb.) was administered intravenously over a period of three minutes. After a reasonably steady state had been attained (usually 20 minutes after injection), levallorphan or nalorphine was given intravenously.




(from Foldes *et al.*, J. Am. M. A., *160*, (1956): 168)

**Graph 1—Chemical structures of the commonly used narcotic analgesics and the N-allyl derivatives.**

Respiratory patterns are shown (graph 1). The change in respiration that accompanied morphine administration was characterized by a decrease in rate and a moderate increase in tidal volume, the net effect being a pronounced decrease in minute volume (table 1). Oxygen consumption showed only a small decrease which, in view of the substantial increase in ventilatory equivalent (ml. oxygen consumed/ml. of ventilation), demonstrates the relatively large capacity for diffusion of oxygen in the dog. Arterial $CO_2$ consistently increased and arterial $O_2$ remained high. Arteriovenous oxygen differences and cardiac output values were erratic and, because of the small number of dogs, no consistent pattern could be demonstrated. Circulatory changes as the result of intravenous morphine administration (graph 2) indicated the presence of a mild hypotension and bradycardia.

The administration of levallorphan or nalorphine resulted in an abrupt reversal of all phases of morphine depression (graph 1, 2; table 1). The minute volume of respiration increased considerably above control levels due primarily to a change in rate and, to a lesser extent, in tidal volume. Ventilatory equivalent decreased markedly. Blood pressure and heart rate were augmented, but cardiac output values varied in either direction without any consistent trend. The content of $CO_2$ in arterial blood decreased markedly. When levallorphan was given in doses of 0.1 mg. per pound of body weight or nalorphine in doses of 0.5 mg. per pound, not only were respiratory circulatory functions enhanced, but also the plane of anesthesia was raised. The administration of levallorphan in doses of 0.05 mg. per pound or nalorphine in doses of 0.25 mg. per pound was followed by an increase in ventilation without any noticeable change in the plane of anesthesia. Five to ten minutes after the antagonist had been given, ventilation decreased but remained above the control levels.

The respiratory and circulatory responses to the slow intravenous administration of meperidine (5 mg./lb.) are shown (graphs 1, 3). Cessation of respiration and a sharp drop in blood pressure occurred as the result of the infusion of only one sixth of the total dose. Three dogs succumbed due to this procedure. Among the dogs that survived, a slow recovery followed, and respiration and circulation became stabilized.

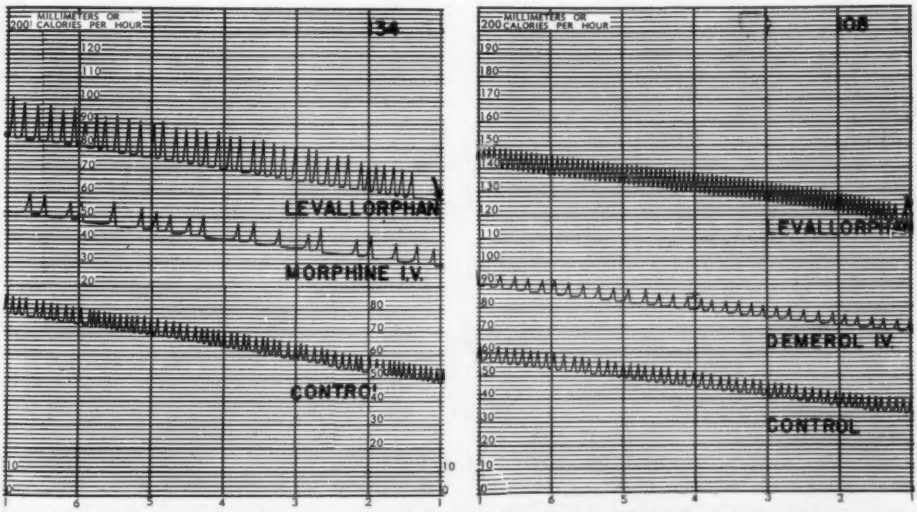
Respiratory depression was the result of a decrease in both rate and tidal volume (table 2). Arterial $CO_2$ content increased, and, in contrast to the effects seen after morphine, cardiac output consistently decreased. The administration of levallorphan or nalorphine reversed all parameters of respiratory and circulatory depression (graphs 1, 3; table 2), leading to (1) an increase in minute volume, (2) a decrease in arterial $CO_2$ content, (3) more rapid restoration of blood pressure toward control levels, and (4) return of cardiac output to control levels. Five to ten minutes after the administration of the antagonist ventilation decreased, but remained above control levels.

**TABLE 2—Effect of Meperidine Administered Intravenously to an Anesthetized Dog, Followed by Levallorphan Administered Intravenously**

| Items * | Pentobarbital alone | Twenty minutes after meperidine | Av. of six minutes after levallorphan |
|---|---|---|---|
| Respiratory rate | 8.90 | 5.90 | 12.90 |
| Tidal volume (cc.) | 141.00 | 125.00 | 151.00 |
| Minute volume (cc.) | 1,222.00 | 763.00 | 1,991.00 |
| Oxygen consumption (cc./min.) | 63.00 | 62.50 | 67.60 |
| Ventilatory equivalent | 0.0516 | 0.0950 | 0.0341 |
| Arterial $CO_2$ (volumes %) | 51.50 | 57.40 | 48.00 |
| Arterial $O_2$ (volumes %) | 20.80 | 18.60 | 20.80 |
| Arterial-venous oxygen difference (volumes %) | 3.10 | 4.40 | 3.20 |
| Cardiac output (liters/min.) | 2.60 | 1.80 | 2.70 |

* Averages of 4 animals.

The administration of levallorphan or nalorphine to dogs anesthetized only with pentobarbital sodium produced a moderate depression of ventilation (graph 4, table 3). In some cases, a small but transient drop in blood pressure also was seen. Such prior administration of the antagonists protected the dogs against the respiratory depression induced by the rapid intravenous administration of large doses of morphine or meperidine. The protection against acute hypotension proved to be unreliable, and no protection against hypotension was afforded the cases illustrated (graph 4). However, none of the dogs in this series, when pretreated with levallorphan or nalorphine, succumbed to the




**Graph 2—Spirograms showing typical respiratory patterns. The space between each vertical line represents one minute. Read from right to left.**

**(134) Control—respiratory pattern during plane 2 surgical anesthesia, induced by pentobarbital sodium; morphine—recorded 20 minutes after the slow intravenous administration of morphine (1.5 mg./lb.); and levallorphan—levallorphan given intravenously at the arrow in a dose ratio of 1 mg. of levallorphan per 50 mg. of morphine.**

**(108) Control—respiratory pattern during plane 2 surgical anesthesia, induced by pentobarbital sodium; Demerol (meperidine)—recorded 20 minutes after the slow intravenous administration of meperidine (5 mg./lb.); and levallorphan—levallorphan given intravenously at the arrow in a dose ratio of 1 mg. of levallorphan per 100 mg. of meperidine.**

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**TABLE 3—Effect of Levallorphan Administered Intravenously to Dogs Not Previously Given an Addicting Analgesic**

| Items | Pentobarbital alone | Av. of six minutes after levallorphan |
|---|---|---|
| Respiratory rate | 12.70 | 10.10 |
| Tidal volume (cc.) | 108.00 | 127.00 |
| Minute volume (cc.) | 1,304.00 | 1,245.00 |
| Oxygen consumption (cc./min.) | 59.70 | 59.70 |
| Ventilatory equivalent | 0.0596 | 0.0593 |
| Arterial $CO_2$ (volumes %) | 52.30 | 53.70 |
| Arterial $O_2$ (volumes %) | 20.50 | 18.50 |
| Arterial-venous oxygen difference (volumes %) | 5.10 | 4.30 |
| Cardiac output (liters/min.) | 1.40 | 1.69 |

rapid intravenous injection of morphine in doses of 1.5 mg. per pound or meperidine in doses of 5.0 mg. per pound.

## DISCUSSION

The interesting pharmacological properties of levallorphan and nalorphine reside in their ability to antagonize with dramatic rapidity all of the depressant effects of morphine, meperidine, and chemically related analgesics.[6, 12, 13, 21, 25] The dog deeply sedated by the narcotic analgesics responded immediately to the intravenous administration of levallorphan or nalorphine. Within 60 to 90 seconds following the administration of the antagonist, the depressant effects of morphine or meperidine largely had been dissipated. A mild degree of sedation persisted, from which the dog was readily aroused. Furthermore, the dosage of levallorphan or nalorphine was regulated so that during pentobarbital sodium anesthesia only the respiratory depressant effects induced by the addicting analgesics were overcome without affecting the level of anesthesia.[11]

Respiratory depression following morphine or barbiturate administration has been shown to result from a decrease in sensitivity of the neural elements that control respiratory activity to changes, particularly increases, in arterial $CO_2$ content.[5] The concept has been advanced that the antagonists restore to the respiratory areas in the brain stem their sensitivity to arterial $CO_2$.[16] Several phases of the experimental data reported here support this hypothesis: (1) The administration of levallorphan or nalorphine to a dog anesthetized with a pentobarbital-morphine or pentobarbital-meperidine combination led to an increase in minute volume greatly exceeding the control levels; (2) ventilatory equivalent declined sharply; and (3) the extreme ventilatory effort subsided as the content of arterial $CO_2$ decreased.

Abnormally high arterial oxygen values were found in a number of experiments following the intravenous administration of the narcotic analgesics. Variations in hematocrit toward hemoconcentration accounted for these exceptional increases in oxygen content. The inspiration of 100 per cent oxygen exaggerated hypoventilation by removing the hypoxic stimulus to respiratory stimulation through the chemoreceptors.[17] Physiological implications of hypoventilation strongly proscribe the use of oxygen therapy without artificial respiration in the presence of depression of the central nervous system.[3] Hypoxia may be averted, but the accumulation of $CO_2$ represents a serious hazard to the maintenance of homeostasis.

Levallorphan and nalorphine, in the doses used, exhibited no analeptic properties. When either was given alone there was some degree of respiratory depression. They did not antagonize barbiturate depression. Evidence has been advanced indicating some analeptic activity by large doses of nalorphine.[24] The data reported here, however, support the opinions of a majority of investigators and clinicians that the usual therapeutic doses antagonize only the activity of the narcotic analgesics.[6, 9, 19]

Attention should be drawn toward the development of acute hypotension following the intravenous administration of morphine or meperidine.[10, 20] It has been shown that morphine,[8] nalorphine,[18] and meperidine [10, 22] are histamine release agents. Histamine release may represent a significant influence in the development of hypotension. The data presented here are compatible with the conclusion that central vasomotor depression also is a major contributing factor in the onset of hypotension.[7] The relatively rapid rate at which the dog's blood pressure, when low, rose after the administration of the antagonist, indicated a central rather than a peripheral site of action. Another factor leading toward the restoration of blood pressure was the increased venous return to the heart as the result of improved respiration. Rapid administration of large amounts of morphine and meperidine (graph 4) produced an acute hypotensive episode which was

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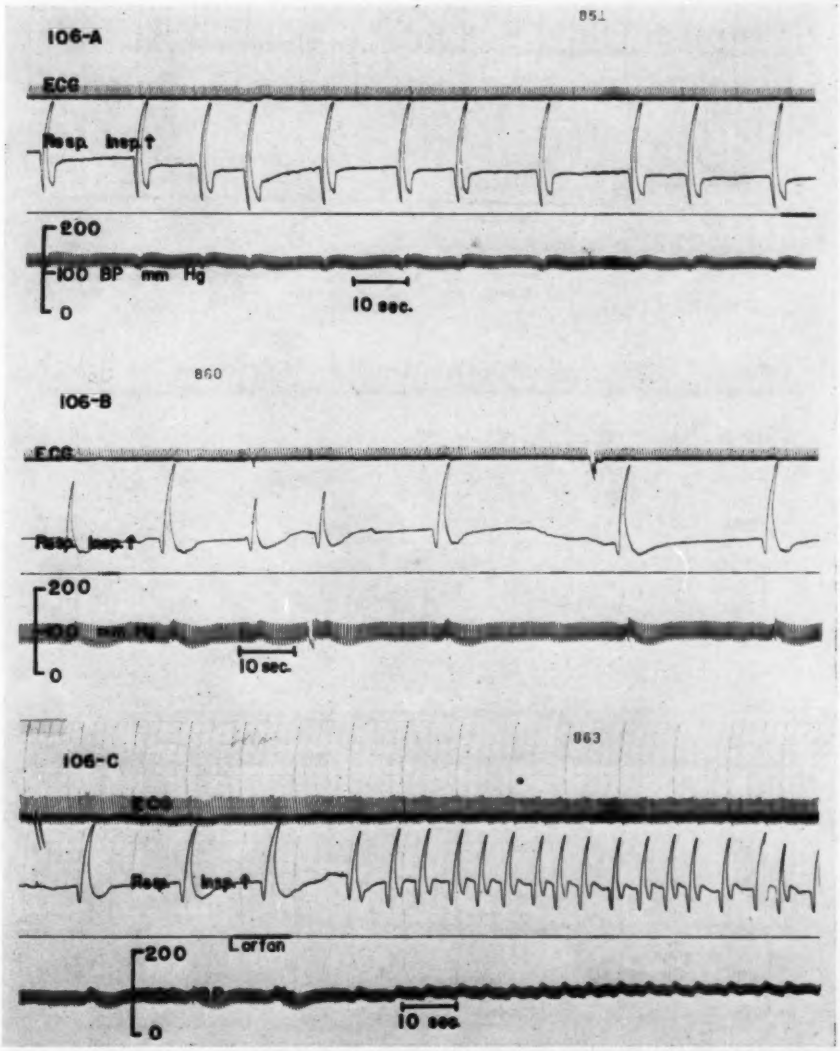
not prevented by the prior administration of levallorphan or nalorphine. Histamine release probably represents the major component in what must be a peripheral vascular response under these conditions.

The mechanism of action of the antagonism between the narcotic analgesics and levallorphan and nalorphine has not been established. The concept of a competitive antagonism is prominent among the theories advanced. Structural similarities between morphine and the antagonists, and the rapidity with which reversal is effected, tend to substantiate this theory of com-




Graph 3—Composite recording showing the effects of intravenously administered morphine, followed by levallorphan, on heart rate, respiration, and femoral arterial blood pressure.

(106-A) Control tracing, plane 2 surgical anesthesia, induced by pentobarbital sodium; (106-B) recorded 20 minutes after the intravenous administration of morphine (1.5 mg./lb.); and (106-C) circulatory and respiratory responses to the intravenous administration of levallorphan (Lorfan) 30 minutes after morphine had been given.

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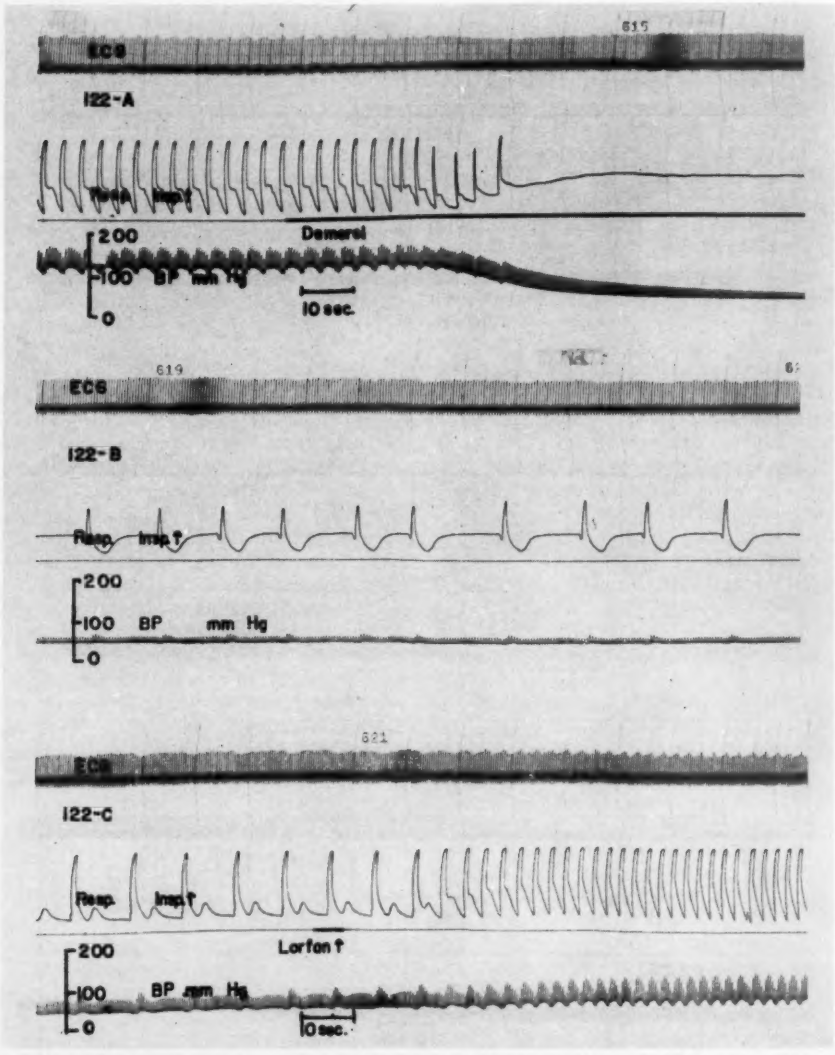
petitive antagonism. The structure-activity relationship of levallorphan and nalorphine to meperidine at first seems obscure. The spatial rearrangement of the meperidine molecule can be shown to have steric similarities to morphine.[14] Thus competitive antagonism may explain the antagonism between meperidine and the narcotic antagonists. A recent study [15] of morphine-nalorphine interrelationships in man has shown that the complex responses may not be compatible with the concept of competi-

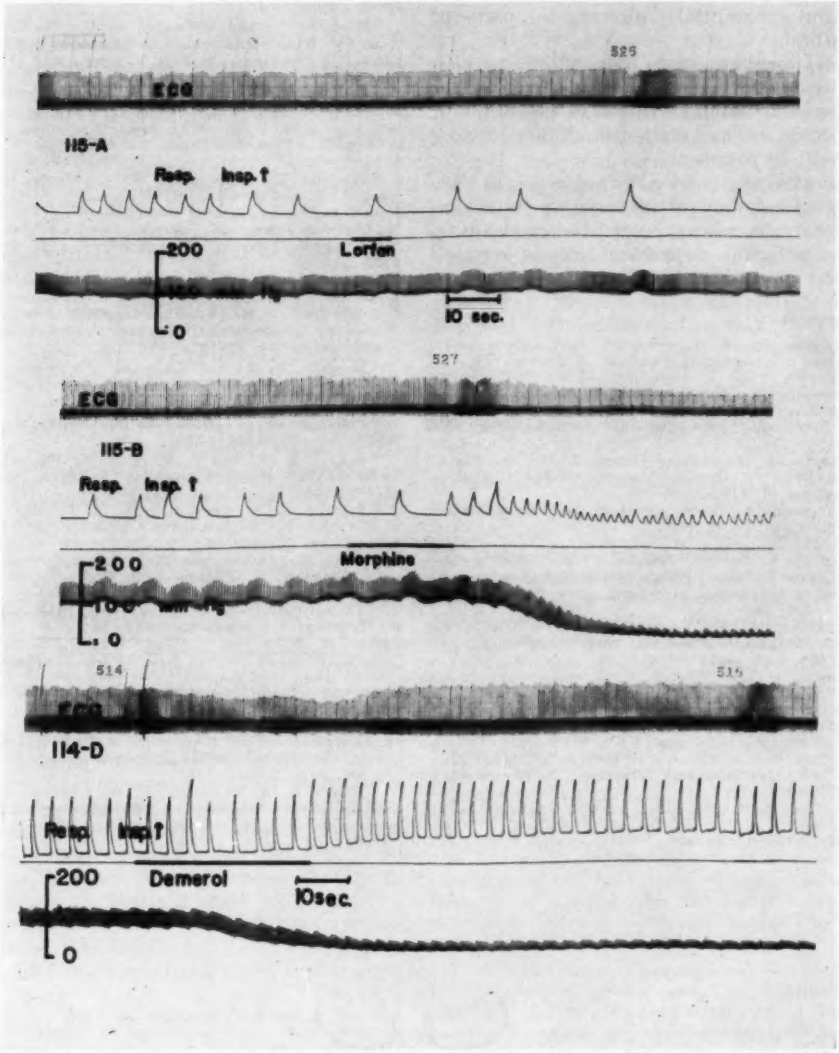



**Graph 4—Composite recording showing the effects of intravenously administered morphine, followed by levallorphan, on heart rate, respiration, and femoral arterial blood pressure. (122-A) Immediate effects of intravenously administered meperidine (Demerol). Notice the cessation of breathing and the sharp drop in blood pressure when only one sixth of the meperidine dose (5 mg./lb.) had been infused; (122-B) recorded 20 minutes after the intravenous administration of meperidine; and (122-C) respiratory and circulatory responses to the intravenous administration of levallorphan 30 minutes after meperidine had been given.**

tive antagonism. The authors have concluded that morphine and nalorphine may affect different receptor sites in the central nervous system rather than exhibiting a competition for the same receptor sites.[15]

## SUMMARY

Levallorphan (Lorfan) tartrate and nalorphine (Nalline) hydrochloride effectively antagonize the sedative effects of morphine and meperidine (Demerol) hy-




Graph 5—Composite recording illustrating various pharmacological properties of levallorphan. (115) Responses to the intravenous administration of 1.0 mg. of levallorphan; (115-B) effect of the rapid intravenous administration of morphine (1.5 mg./lb.) to a dog previously given 1.0 mg. of levallorphan—notice the sharp drop in blood pressure and the conversion of the slow deep respirations to a panting type of breathing; and (114-D) effect of the rapid intravenous administration of meperidine (Demerol), 5.0 mg./lb., to a dog previously given 1.0 mg. of levallorphan.

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drochloride). During combined barbiturate-morphine or barbiturate-meperidine anesthesia, appropriate doses of levallorphan or nalorphine were associated with a rapid increase in ventilation, a decrease in arterial $CO_2$ content, and a return of blood pressure, when low, toward normal levels, without substantially altering the plane of anesthesia.

Levallorphan and nalorphine prevent the respiratory depression induced by the intravenous administration of morphine or meperidine. The antagonists do not reliably prevent hypotension.

Levallorphan and nalorphine per se have central nervous depressant properties. These drugs are not analeptics, and antagonize only the depressant effects induced by the narcotic analgesics.

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# Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok Antelope, Using Glycine-2-$C^{14}$

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THE ESTIMATION of erythrocyte survival time within the suborder Ruminantia has been limited to techniques utilizing $Cr^{51}$-tagged [11, 12] and $Fe^{59}$-labeled [1] erythrocytes in domestic sheep and cattle. Mean erythrocyte survival time has been estimated by Baker and Douglas [1] to be 56, 52, and 54 days in 3-month-old lambs, yearling lambs, and 3-month-old calves, respectively.

Due to problems encountered in the *in vivo* elution of chromium-51 from the circulating erythrocytes [16] and from the indirect nature of the ferrokinetic method,[1] it appeared desirable to determine the survival time by the use of a direct method using carbon-14. In the present study, ruminant erythrocytes were labeled with radioactivity by the injection of glycine-2-$C^{14}$, from which the $\alpha$-methylene carbon atom is incorporated into 8 of the 34 carbon atoms of the porphyrin moiety of hemoglobin. Glycine-2-$C^{14}$ has been used previously in similar studies in human beings,[17] rats,[14] and swine.[6]

The term "life span" has been referred to by other investigators [5, 6] as the longevity of the erythrocyte population when death is attributed to senescence. In addition to the process of senescence in some mammals, the effects of random destruction of erythrocytes, common to nucleated cells, have been reported to be present.[5, 6] Random destruction of erythrocytes may also accompany certain pathological diseases.[3] In studies in which the survival of the erythrocyte population is a more complex process, erythrocyte longevity has been termed the "survival time."[5] The methods presently used for the estimation of erythrocyte longevity have been critically analyzed recently by Eadie and Brown.[9]

The majority of investigators have estimated erythrocyte life span by a mathematical treatment recommended by Shemin and Rittenberg.[17] These investigators essentially computed the time after formation of a cell population at which the rate of destruction was maximum. This statistic appears to be quite sensitive to minor changes in the experimental data and is not well defined when the maximum rate of destruction of erythrocytes is constant for an extended time. In the present investigation, we computed the time, after the formation of a cell population, at which one half of the cells still survived.

## MATERIALS AND METHODS

Ungulates from two families, Bovidae and Cervidae, were chosen from the suborder Ruminantia. In Bovidae, a 19-month-old female springbok antelope *(Antidorcas marsupialis)* from the tribe Antilopini, and a 10-month-old female aoudad or Barbary sheep *(Ammotragus lervia)* from the tribe Caprini, were selected. In Cervidae, a 6-month-old female mule deer *(Odocoileus hemionus)* from the tribe Odocoileini, was used. All experimental animals were caged at the San Diego Zoo.

The experimental animals were inoculated intravenously with glycine-2-$C^{14}$ and weekly blood samples were taken for the isolation of hemin. Glycine-2-$C^{14}$ was injected as follows: 27.6-kg. springbok antelope, 18.5 $\mu$c./kg.; 32.0-kg. mule deer, 15.6 $\mu$c./kg.; and 45.5-kg. aoudad sheep, 11.0 $\mu$c./kg. During the latter part of the trial, tranquilizers* (2 ml.) were necessary prior to taking blood samples from the aoudad sheep and mule deer. Complete erythrocyte counts were performed weekly.

Twenty milliliters of venous blood was used for the isolation of crude hemin as described by Fischer [10] and recrystallized and purified in the manner described by Shemin and Rittenberg.[17] Additional recrystallization of the hemin did not increase its specific activity. Hemin samples were burned to $CO_2$ according to Van Slyke and Folch.[21] Planchets of $Ba^{14}CO_3$ were prepared from the combusted samples as described by Kleiber and Edick [15]

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* Diquel, produced by Jensen-Salsbery Lab., Kansas City, Mo.



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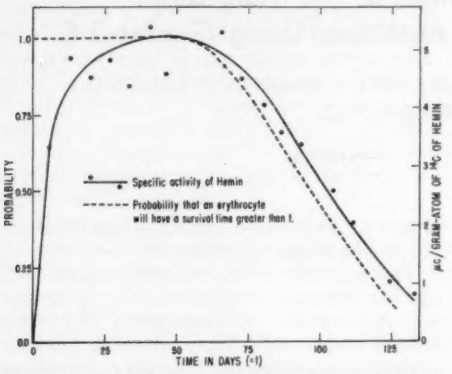

PROBABILITY
TIME IN DAYS (±1)
μc/GRAM-ATOM OF ¹⁴C OF HEMIN
Specific activity of Hemin
Probability that an erythrocyte will have a survival time greater than t.

**Graph 1—Probability of erythrocyte survival and specific activity of hemin in the mule deer following the injection of glycine-2-C$^{14}$.**

and counted for radioactivity at infinite thickness with a Tracerlab windowless geiger flow gas counter and "1,000" scaler. A correction for the amount of erythrocyte radioactivity removed by sampling was made by the method of Valentine *et al.*[20]

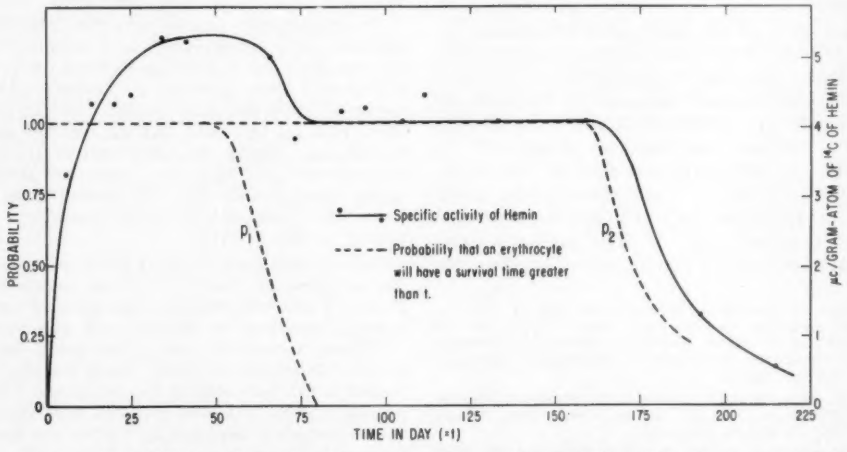
## DISCUSSION

All of the important information concerning the survival times of the erythrocytes is contained in the function $p(t)$, where $p(t)$ is the probability that an erythrocyte will have a survival time greater than $t$. The function $p(t)$ can be interpreted as the fraction of a cell population remaining $t$ days after its formation. These probability functions are shown graphically for each of the experimental animals (graphs 1–3). The primary difficulty in computing $p(t)$ arises from the fact that the initial labeling of cells occurs over a considerable time. As previously indicated by Shemin and Rittenberg,[17] $p(t)$ satisfies an integral equation of the form:

$$(1) \qquad C(t) = \int_0^t R(\theta)\, p(t-\theta)\, d\theta,$$

where $C(t)$ is the C$^{14}$ concentration at time $t$ and $R(\theta)$ is the gross rate of change of concentration of C$^{14}$ at time zero; *i.e.*, $R(\theta)$ takes account of production of new radioactive heme at time zero, but does not take account of any destruction of radioactive heme. Equation 1 is an integral equation of the first kind of convolution type. If the functions $C$ and $R$ are known, the function $p$ can generally be determined by Laplace transform methods.[8]

The function $C$ is known directly from the experimental data, while the function $R$ must be computed indirectly. The method of Shemin and Rittenberg[17] consists in finding an approximation for $C(t)$, which is valid for values of $t$ up to about 30 days. Since it has been shown by Cornelius *et al.*[7] that the specific activity of C$^{14}$-labeled glycine falls off exponentially after its intravenous injection into ruminants, the continuous synthesis of heme should not continually contribute significant radioactivity to the circulating heme pool. The isotope concentration in the free glycine pool of cows, 48 hours after injection, was


PROBABILITY
TIME IN DAY (±1)
μc/GRAM-ATOM OF ¹⁴C OF HEMIN
Specific activity of Hemin
Probability that an erythrocyte will have a survival time greater than t.
p1
p2

**Graph 2—Probabilities of erythrocyte survival and specific activities of hemin in an aoudad sheep following the injection of glycine-2-C$^{14}$.**

found to be 0.5 per cent of the isotope concentration during the first hour. If we assume in the present study that no cell destruction occurs up to 30 days, then $p(t) = 1$ for $t$ less than 30 days. Thus, the gross rate of change of concentration of $C^{14}$ is equal to the net rate of change, and we have for $t$ less than 30 days:

$$R(t) = \frac{d}{dt} C(t). \tag{2}$$

If we find an approximation for $C(t)$ of the form

$$C(t) = C_o (1 - e^{-\lambda t}) \tag{3}$$

we are led to the approximation:

$$R(t) = C_o \lambda e^{-\lambda t} \tag{4}$$

For values of $t$ greater than 30 days, this approximation is still used, since in that region, $R(t)$ is known to be negligibly small.

Using the methods of Laplace transformation [8] or those suggested by Shemin and Rittenberg,[17] one finds

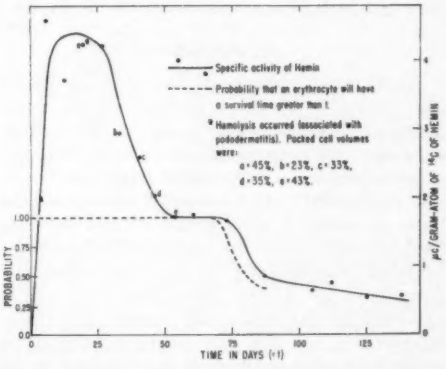
$$p(t) = \frac{1}{C_o}\left( C(t) + \frac{1}{\lambda}\frac{dc}{dt} \right). \tag{5}$$

In the aoudad sheep, $C(t)$ exhibited two distinct plateaus rather than one plateau before dropping off to near background radioactivity. This was interpreted as showing the existence of two separate erythrocyte populations with differing survival times. Let us assume that at any moment during the initial labeling period (approx. the first 30 days), the specific activity in the heme of both populations is the same. We attribute the first decrease in $C(t)$ to the death of the first population, and the second decrease to the death of the second population. Under these assumptions, we can write $C(t)$ as a sum

$$C(t) = C^1(t) + C^2(t) \tag{6}$$

where $C^1(t)$ takes account only of the activity of the first population and $C^2(t)$ of the second population. It is then possible to compute the corresponding probability functions $p_1(t)$ and $p_2(t)$ for the component populations using the method previously described. Let $C_o^1$ and $C_o^2$ denote the maximums of $C^1(t)$ and $C^2(t)$, respectively. Then, under the assumptions given previously, and the assumption that the amount of heme contained in an erythrocyte is the same for both populations, the ratio $C_o^1 : C_o^2$ is just the ratio of the number of cells in the first population to the number of cells in the second population. We would conclude, therefore, that about 20 per cent of the erythrocytes belong to the first population.

Since it is useful to give some simple statistic to describe the survival time, we have calculated for each probability function the time $t_{1/2}$ is such that $p(t_{1/2}) = \frac{1}{2}$. These numbers are referred to as "half-survival times" of the various cell populations. However, it must be emphasized that, in using this term, we are not asserting that a process similar to radioactive decay is present. The evidence presented here appears to indicate a more complicated process, in which the survival of a cell depends on its past history.


Specific activity of Hemin
Probability that an erythrocyte will have a survival time greater than t
Hemolysis occurred (associated with pododermatitis). Packed cell volumes were:
a - 45%, b - 23%, c - 33%,
d - 35%, e - 43%.
PROBABILITY
μc/GRAM-ATOM OF 14C OF HEMIN
TIME IN DAYS (-t)

**Graph 3—Probability of erythrocyte survival and specific activities of hemin in a springbok antelope following the injection of glycine-2-$C^{14}$.**

It was of special interest that the aoudad sheep used in the present study appears to be the first apparently normal mammal found to have two distinctly separate populations of erythrocytes. The half-survival times of these two populations were 65 and 170 days, respectively. Berlin *et al.*[2] have described the presence of a second population of cells with a short survival time in patients with polycythemia vera. These immature cells of the erythroid series were observed in addition to the cells surviving normally. However, in the aoudad sheep, no morphological differences in erythrocytes were observed. Huisman *et al.*[13] recently reported that the aoudad sheep possesses a different type of hemoglobin, with its own chemical and physiological properties, than that observed in other

sheep. That variations in the survival time of erythrocytes may occur from differing types of hemoglobins has been well established in cases of thalassemia major,[14] sickle cell anemia,[18] and the presence of homozygous type iii (c) hemoglobin[19] in man. Unpublished data from our laboratory, using glycine-2-$C^{14}$, indicate that erythrocytes of domestic sheep possess shorter survival times than those of the aoudad sheep.

Unfortunately, during the investigation the springbok antelope was affected by a hemolytic process which was associated with a septic pododermatitis. Following recovery from the anemia, a constant $C(t)$ existed in the erythrocytic heme; therefore, an estimate of an 80-day half-survival time was made. The erythrocytes of the mule deer had a half-survival time of 95 days.

## SUMMARY

Erythrocyte survival was studied using glycine-2-$C^{14}$ in three ruminants: a mule deer, an aoudad sheep, and a springbok antelope. The survival of the erythrocyte populations was expressed graphically as the probability that an erythrocyte would have a survival time greater than $t$. The half-survival time for the erythrocyte populations was defined as the time after the formation of a cell population, at which one-half of the cells still remained. Half-survival times for the erythrocytes of a mule deer and springbok antelope were 95 and 80 days, respectively. An aoudad sheep exhibited two separate populations of erythrocytes, with half-survival times of 65 and 170 days, respectively.

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# Studies of Goat Serum Proteins by Paper Electrophoresis

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THE UTILIZATION of goats as research study animals made evident the fact that few recent data were available pertaining to goat serum protein normal concentrations, distribution of components, and their respective concentrations.

The work of Reineke *et al.*, in 1939,[4] was found to be the only available study of substance pertaining to this subject. This study dealt with sodium sulfate salting-out procedures employing dairy goat plasma; in only two instances were goat serum samples utilized. The salting-out technique employed indicated the presence of three major components in the globulin fraction of goat serum. Consideration was also given to certain small solubility breaks with these workers considered as representing divisions of pseudoglobulin I and pseudoglobulin II.

It was felt that paper electrophoresis partition of goat serum might be of interest and might help to resolve some of the problems usually encountered in solubility salting-out procedures of proteins.

This paper will deal with paper electrophoresis partition studies of normal goat serum and will encompass the following: *(a)* determination of total protein concentrations of normal goat serums; *(b)* paper electrophoresis partition of goat serum specimens; *(c)* determination of the respective concentrations of protein entities, *i.e.*, albumin, globulin, and globulin components; and *(d)* identification of goat serum protein components according to Rf values with reference to Rf values of human serum protein components.

## MATERIALS AND METHODS

The test group of animals consisted of 15 goats from 7 to 9 months old, and were of various recognized breeds excluding the Angora variety. They were maintained at Dallas, Texas, on yellow-blue stem grass pasture, plus oak leaves, acorns, and briar. There was no supplemental feeding.

The does were not pregnant during the course of the experiment. The animals were examined for parasites prior to beginning the study and were considered free of internal parasites; consequently, no anthelmintic was given. There were no manifestations of external parasites. Animals selected were separated from the others of the flock.

The 15 goats, 10 females, 3 entire males, and 2 wethers, were numbered for identification and further subdivided into three groups of 5 animals each. The groups were designated as control group 1, and groups 2 and 3, to aid in further studies which will be the subject of a later report.

The initial blood sampling included all 15 animals. Blood specimens were obtained from the jugular vein and allowed to clot. Serum was pipetted off and centrifuged clear at 3,000 r.p.m. The numbered samples were placed on dry ice, packaged, and shipped by air to the laboratory for study.

The control group animals were sampled again three weeks later. Twenty serum samples were used to establish normal distribution of total serum protein concentration as well as serum components. Although limited in number, it was felt that this method of sampling would supply the lowest number which would have statistical significance.

Total serum protein concentration determinations were made by the biuret method of Kingsley.[3] The method was standardized employing an assayed chemical control serum.*

Paper electrophoresis separations of 0.008-ml. serum samples, applied by a striper, were accomplished in a Durrum-type cell,† using a barbital buffer of pH 8.6, ionic strength 0.075.

A running time of 16 to 18 hours with a current setting of 8 ma. was used for the separation. Protein staining was accomplished with bromphenol blue dye by the method of Block *et al.*[2]

Stained strips were scanned and integrated by employing the Analytrol recording scanner and integrator.[1]

The Rf values were calculated using point of application of serum sample to paper strip as the base and measuring the distance traveled by each respective component. This was compared to the full distance traveled by the albumin fraction.

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* Hyland lot #369D1, supplied by Hyland Laboratories, Los Angeles, Calif.

† Manufactured by the Spinco Division, Beckman Instruments Inc., Belmont, Calif.



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**TABLE 1—Goat Serum Protein Component Distribution and Concentration**

| Goat No. | Sex | Total protein (Gm./100 ml.) | Albumin | *Alpha* | *Beta*-1 | *Beta*-2 | *Gamma* |
|---|---|---|---|---|---|---|---|
| | | | | (Concentration in % of total protein) | | | |
| 59–1 | F | 6.3 | 63.0 | 5.0 | 14.5 | .... | 17.5 |
| *59–2 | F | 6.9 | 63.5 | 5.5 | 13.5 | .... | 17.5 |
| 66–1 | F | 6.1 | 56.0 | 7.0 | 14.0 | .... | 23.0 |
| *66–2 | F | 6.1 | 64.5 | 6.5 | 9.5 | .... | 19.5 |
| 67–1 | F | 6.3 | 68.0 | 5.5 | 12.0 | .... | 14.5 |
| *67–2 | F | 6.3 | 70.5 | 5.0 | 8.5 | .... | 16.0 |
| 69–1 | F | 6.5 | 60.0 | 5.0 | 14.5 | .... | 20.5 |
| *69–2 | F | 6.7 | 59.0 | 8.5 | 15.0 | .... | 17.5 |
| 73–1 | F | 6.5 | 56.0 | 7.0 | 11.0 | 8.5 | 17.5 |
| *73–2 | F | 5.7 | 68.5 | 10.0 | 10.0 | 6.5 | 5.0 |
| 74–1 | F | 6.3 | 66.0 | 8.5 | 10.5 | 5.5 | 9.5 |
| 75–1 | F | 6.8 | 70.5 | 4.5 | 12.0 | .... | 13.0 |
| 63–1 | F | 6.3 | 67.5 | 5.5 | 8.0 | 6.0 | 13.0 |
| 64–1 | F | 6.3 | 63.0 | 7.5 | 14.0 | .... | 15.5 |
| 65–1 | F | 5.8 | 65.5 | 7.0 | 13.0 | .... | 14.5 |
| 60–1 | M | 5.8 | 66.5 | 5.0 | 14.5 | .... | 14.0 |
| 61–1 | M | 6.3 | 60.5 | 4.5 | 19.0 | .... | 16.0 |
| 62–1 | M | 5.7 | 59.0 | 8.0 | 17.5 | .... | 15.5 |
| 68–1 | W | 6.5 | 68.0 | 5.0 | 14.5 | .... | 12.5 |
| 71–1 | W | 6.0 | 51.0 | 14.0 | 19.0 | .... | 16.0 |

* Goats 59, 66, 67, 69, and 73 were of the control group and appear twice in the tabulations.

Serum samples from human beings were run concurrently and the calculated Rf values of goat serum components compared to the Rf values obtained for serum components of man.

## RESULTS

The determinations of total protein concentrations (table 1) were run in duplicate to determine reproducibility. All were found to be within ± 3.5 per cent limits. The tabulation of the respective albumin and globulin component values was based on scanning and integration of the duplicate strips, separated concurrently. These were found to reproduce within a maximum deviation of ± 1.8 per cent in any single component.

Variations were encountered in the chemical and electrophoretic studies of the control group at the second sampling. Serums of goats 67 and 69 showed a maximum variation of 4.5 per cent in any single component. The serum specimens of goats 59 and 66 showed greater variations; the former a 9.2 per cent change in the total protein concentration and the latter an 8.5 per cent change in the albumin entity. Goat 73 showed the greatest variation, ranging to 12.5 per cent. Reasons for these variations from the initial determinations were not established.

The statistical evaluation of the data in table 1 is presented (table 2).

In a comparison of the mean concentrations of the various protein entities, according to the sex of the animal (table 3), the presence of the *beta*-2 fraction in the female is of interest. Occurrence of this fraction in both serum specimens of control goat 73 appears to establish this as an entity and not a possible artefact due to technique, paper, or contamination of serum (strip 4, fig. 2).

To establish significance of sex on the entire group mean values, percentile differences of components of each sex group are

**TABLE 2—Statistical Evaluation of Data Tabulated in Table 1**

| Item | Concentration (Gm./100 ml.) | | | | | |
|---|---|---|---|---|---|---|
| | | | Globulins | | | |
| | Total protein | Albumin | *Alpha* | *Beta*-1 | *Beta*-2 | *Gamma* |
| Range | 5.70 | 3.05 | 0.30 | 0.50 | 0.35 | 0.30 |
| | 6.90 | 4.80 | 0.85 | 1.20 | 0.55 | 1.40 |
| Mean | 6.25 | 3.95 | 0.42 | 0.83 | 0.41 | 0.97 |
| Standard deviation | ± 0.35 | ± 0.26 | ± 0.09 | ± 0.12 | ± 0.07 | ± 0.18 |
| Percentage variation | 5.3 | 6.5 | 21.4 | 14.5 | 17.0 | 18.6 |
| Av. percentage concentration | ..... | 63.3 | 6.7 | 13.2 | 6.6 | 15.5 |
| Range of percentage concentration | ..... | 51.0–70.5 | 4.5–14.0 | 8.0–19.0 | 6.0–8.5 | 5.0–23.0 |

$$\text{Percentage variation} = \frac{100 \times \text{s.d. of component}}{(M)}, \quad M = \text{mean concentration of respective component.}$$

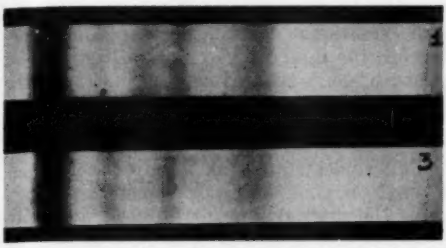


Fig. 1—Comparison of electrophoresis partition of human serum (strip 1) and goat serum (strip 3), showing the predominant three-fraction globulin component separation. Notice the relative mobilities, as well as number, of the alpha- and beta-globulin fractions in both specimens.

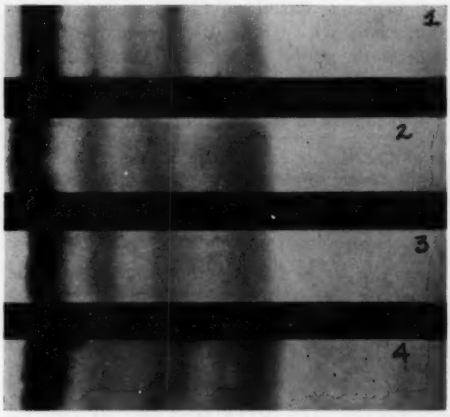


Fig. 2—Comparative study of human serum (strip 1) and goat serum (strips 2, 3, and 4) separations, showing the number and mobilities of the various globulin fractions. The four-fraction globulin in strip 4 shows beta-1 and beta-2 entities.

shown (table 3). Included is the group mean of the second sampling of the control group and variations of its components from the entire group mean. Deviations from entire group mean were found to be within acceptable limits with a maximum change of 5.5 per cent.

The statistical significance of the *beta*-2 fraction can not be evaluated due to the limited number of appearances. Consequently, it has been omitted from consideration in the 90 per cent confidence limits, and the male and wether groups of goats.

The relative Rf values of the various globulin fractions of goat serum as compared to Rf values of the globulin fractions found in serum of man are presented (table 4). The relative mobilities of albumin and *gamma* globulin (fig. 1, 2) are similar in both human and goat serum specimens. The data in table 4 show the relative mobilities of the *alpha* and *beta* globulins, as well as the number of respective fractions, in the goat serum vary from those found in serum of man. Consequently, only these were compared in the Rf calculations.

## DISCUSSION

The work of Reineke *et al.*[4] resulted in the establishment in the globulin fraction of goat serum of euglobulin, pseudoglobulin I, and pseudoglobulin II. Other conclusions drawn by these workers were that pseudoglobulin I and pseudoglobulin II were composed of two subfractions each.

The pioneer work of Tiselius[5] has resulted in this nomenclature being replaced by the now standard terms: *alpha*, *beta*, and *gamma* globulins, with the *alpha* globulin fractions being designated as *alpha*-1

TABLE 3—Comparison of Group Means According to Sex, Percentile Variations of Components from Entire Group Means, and 90 Per Cent Limits

| Item | Mean total protein (Gm./100 ml.) | Albumin | *Alpha* | *Beta*-1 | *Beta*-2 | *Gamma* |
|---|---|---|---|---|---|---|
| | | (Concentration in % of total protein) | | | | |
| Females (10) | 6.30 | 64.2 | 6.4 | 13.1 | 5.7 | 16.5 |
| Percentage deviation of component from group mean | +1.0 | +0.9 | −0.3 | −0.1 | −0.9 | +1.0 |
| Females (5) second sampling control group | 6.35 | 65.2 | 7.1 | 11.3 | 4.7 | 15.3 |
| Percentage deviation of component from group mean | +0.9 | +1.9 | +0.4 | −1.9 | −1.9 | −0.2 |
| Wethers (2) | 6.25 | 60.0 | 9.4 | 16.6 | .... | 14.2 |
| Percentage deviation of component from group mean | −0.0 | −3.3 | +2.7 | +3.4 | .... | −1.3 |
| Males (3) | 5.90 | 65.0 | 5.8 | 17.3 | .... | 14.7 |
| Percentage deviation of component from group mean | −5.5 | +1.7 | −0.9 | +4.1 | .... | −0.8 |
| 90 per cent limits entire group | 5.7–6.7 | 53.0–69.0 | 4.5–9.0 | 8.5–17.5 | .... | 10.0–23.0 |

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and *alpha*-2, based on mobility in electrophoresis work. Consequently, all comparisons were made using this nomenclature.

**TABLE 4—Comparison of Rf Values of Globulin Components in the Serum of Goat and Man**

| Serum | Globulin component | | | | | |
|---|---|---|---|---|---|---|
| | *Alpha*-1 | *Alpha* | *Alpha*-2 | *Beta*-1 | *Beta* | *Beta*-2 |
| Man | 0.75 | ..... | 0.53 | ..... | 0.29 | ..... |
| Goat | ..... | 0.71 | ..... | 0.37 | ..... | 0.25 |

At no time during the course of this phase of the study of goat serums was there evidence of two fractions in the *alpha* globulin entity. Two fractions, of varying mobilities, in the *beta* globulin entity occurred in 20 per cent of the electrophoretic separations and were confined to the females. This agreed in part with the work of Reineke *et al.*[4]

The usual three-fraction globulin separation and the occasionally encountered four-fraction separation are shown (fig. 2). The four-fraction separations of the globulin component in the serum occurred in only 3 goats representing 15 per cent of the normal group. This low incidence would indicate that the three-fraction globulin separation is normally encountered in goat serum upon paper electrophoresis fractionation.

Of interest were the variations in the mobility of the various globulins as compared to those found in serum of man. It was felt by the authors that utilization of the presently available nomenclature would suffice in further studies of goat serum.

No comparison is made between results of the sodium sulfate salting out procedures used by Reineke *et al.*[4] and the electrophoresis partition of goat serum, since it is well known that each technique has certain inherent variables. The wide span in the normals of the various protein components and fractions cannot be explained, although the mixed breed composition of the group of goats studied may in some measure be responsible.

## SUMMARY

Paper electrophoresis studies were conducted on goat serum specimens to determine the normal concentration of albumin, globulin, and globulin fractions, as well as the number and mobilities of the various protein components.

Total proteins were determined by a chemical method.

The presence of four fractions in the globulin component, which were confined to the female of the species, agreed in part with the work of earlier investigators employing chemical salting-out techniques. Two fractions were found in the *beta* globulin component and were identified as *beta*-1 and *beta*-2, on the basis of comparison with Rf values of human serum component mobilities.

Mobility of the *alpha* globulin was found to be between the *alpha*-1 and *alpha*-2 fractions of human serum.

Three-fraction separation in the globulin component was found to occur in the majority of electrophoresis patterns.

### References

[1] Beckman Instruments Inc., Belmont, Calif.; Spinco Division: Spinco Model R., Paper Electrophoresis Operating Instructions, OIR-1.

[2] Block, R. J., Durrum, E. L., and Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York, 1955.

[3] Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry. 12th ed. Blakiston Co., Philadelphia, Pa. (1949): 550–553.

[4] Reineke, E. P., Peterson, V. E., and Turner, C. W.: The Partition of Serum Globulins of the Dairy Goat. J. Biol. Chem., *128*, (1939): 1–7.

[5] Tiselius, A.: Electrophoretic Analysis and the Composition of Native Fluids. Harvey Lectures, *35*, (1939–1940): 37. J. P. Lippincott Co., Philadelphia, Pa.

# Experimental Brucellosis in White-Tailed Deer

W. G. YOUATT, B.S., and L. D. FAY, D.V.M., M.S.

*East Lansing, Michigan*

IN CONJUNCTION WITH a recent survey [2] to determine the incidence of brucellosis in Michigan white-tailed deer *(Odocoileus virginianus)*, the Michigan Department of Conservation artificially infected 2 deer with *Brucella abortus*. The purpose was to find out if deer would develop a titer against *Br. abortus* antigen, to what levels the titer might rise, how long a titer might persist, and if the reaction of positive deer serum would be similar to that of positive bovine serum. Also, 2 fawns were vaccinated with *Br. abortus* vaccine, strain 19.

## MATERIALS AND METHODS

The deer used in this experiment were kept in pens at the Game Division Laboratory at the Rose Lake Wildlife Experiment Station near East Lansing, Mich. Preinoculation tests of all 4 deer were negative for brucellosis.

The 2 male deer, 1½ years old, were inoculated with a virulent 48-hour culture of *Br. abortus*, strain 2308. This was a suspension of 10 million organisms per 0.1 ml. of a 10 per cent peptone solution. Each deer was inoculated with 0.05 ml. of the suspension in the conjunctival sac of each eye, a total of 10 million organisms per deer. The culture was supplied by the Animal Disease Control and Eradication Division, Animal Research Service, U. S. Department of Agriculture. The 2 male fawns, 6 months old, were each vaccinated subcutaneously with 5.0 ml. of commercially prepared* *Br. abortus* vaccine, strain 19.

Serum samples were obtained at intervals ranging from daily to several weeks, depending upon the anticipated and preceding reactions of the animals to inoculation or vaccination. In testing the serums for titers, the standard tube-agglutination test was employed, using tube-type *Br. abortus* antigen supplied by the U.S.D.A.

## RESULTS

Serum from adult deer 1, inoculated in October, 1957, first showed a titer at three weeks; that of adult deer 2, inoculated in December, 1957, at two weeks (fig. 1). In deer 1, the titer reached a high of 1:1,600 at 32 weeks after inoculation and remained at this level for 20 weeks, when the experiment was concluded (Oct., 1958). The titer of deer 2 rose irregularly to a high of 1:3,200 at 26 weeks after inoculation and maintained this level until the end of the experiment 20 weeks later.

Both fawns, vaccinated in December, 1957, showed an initial reaction two days after vaccination (fig. 2). Their titers rose to a high of 1:1,600 seven days after vaccination and then gradually receded. The titers leveled off at 1:50 and 1:25 at 15 and 24 weeks after vaccination, respectively, with the exception of the determination at 29 weeks for fawn 2. They did not rise higher than this through the remainder of the experiment. These results are similar to those encountered with vaccinated calves.[1]

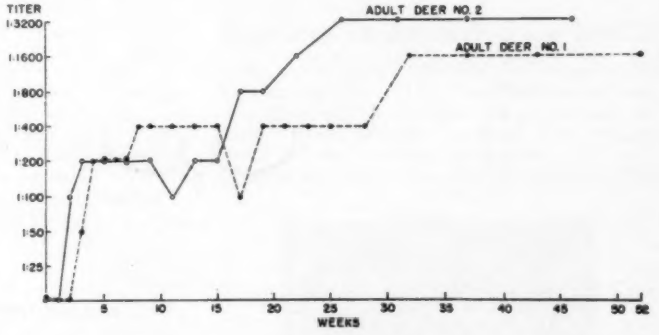


**Fig. 1—Titers of deer inoculated with Brucella abortus, strain 2308.**

We compared Brucella-positive deer serum with Brucella-positive bovine serum,

From the Michigan Department of Conservation, Game Division Laboratory, Rose Lake Wildlife Experiment Station, East Lansing.

* Jensen-Salsbery Laboratories, Inc., Kansas City, Mo.



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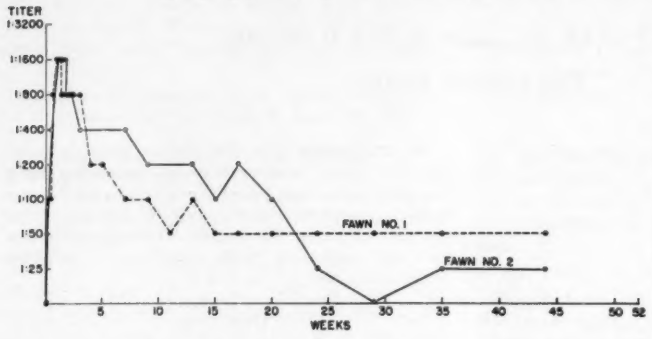


**Fig. 2—Titers of fawns vaccinated with Brucella abortus vaccine, strain 19.**

using the plate-agglutination method, and found the reactions to be identical. This enabled us to use the plate-agglutination test with confidence on our survey of brucellosis in deer.

## SUMMARY AND CONCLUSIONS

White-tailed deer will develop and maintain a demonstrable titer to *Brucella abortus*. The plate-agglutination test can be used to detect *Br. abortus* antibodies in deer serum; the reaction is identical to that encountered with bovine serum. Although it is possible to infect deer with *Br. abortus* experimentally, we do not know if they are as susceptible as cattle to infection in the natural state. Vaccinated fawns show an antibody response typical of that found in vaccinated calves.

### References

[1] Manthei, C. A.: Evaluation of Vaccinal Methods and Doses of *Brucella Abortus* Strain 19. Proc. U.S. Livestock San. A., 56th Ann. Meet., 1952.

[2] Youatt, W. G., Fay, L. D., Whitehead, G. L., and Newman, J. P.: Brucellosis and Leptospirosis in White-Tailed Deer in Michigan. J. Wildlife Mgt., *23*, (1959): 345–348.

# Scientific Notes

## Instrument Symposium and Research Equipment Exhibit

The Ninth Annual Instrument Symposium and Research Equipment Exhibit, sponsored by research equipment manufacturers and six scientific societies, will be held September 28 through October 1 at the National Institutes of Health, Bethesda, Md.

Displays will feature the latest developments in electronics, laboratory glassware, and surgical, optical, radiation, and gas-sampling instruments. Demonstrations in gas chromatography and electromagnetophoresis will be staged; a symposium on gas chromatography and a lecture on electromagnetophoresis are included in the program.

In addition to a panel discussion of instrumentation and special instrument problems, speakers will present papers on serum agar methods, irradiation of cells in tissue culture, and nuclear and electronic magnetic resonance.

Additional information is available from James B. Davis, National Institutes of Health, Public Health Service, Bethesda 14, Md.

## Animal Care Panel Meeting

The Tenth Annual Animal Care Panel Meeting will be held Oct. 29–31, 1959, at the Sheraton Park Hotel, Washington, D.C.

In addition to the scientific and commercial exhibits, a number of movies on laboratory animal care will be shown continuously each day. The formal program will be divided into six sessions concerning genetics, housing and management, nutrition, equipment, germ-free and specific pathogen-free animals, and disease. Four papers will be presented on each of these subjects.

For additional information, contact William I. Gay, Animal Care Panel, 2101 Constitution Ave., N.W., Washington 25, D.C.

## Symposium on Antibiotics

The Seventh Annual Symposium on Antibiotics, sponsored by the journals *Antibiotics & Chemotherapy* and *Antibiotic Medicine & Clinical Therapy*, will be held Nov. 4–6, 1959, at the Mayflower Hotel, Washington, D.C.

Papers presented at this meeting will be published in "Antibiotics Annual 1959–1960." Authors interested in contributing may obtain information on manuscript preparation by contacting Dr. Henry Welch, Director, Division of Antibiotics, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington 25, D.C.

For additional information and pre-registration cards, write to Dr. Welch.

## Southern Conference on Avian Diseases

A Southern Conference on Avian Diseases was organized April 2, 1959, at Raleigh, N. Car., during the annual meeting of the Animal Disease Research Workers in the Southern States. It is affiliated with and will function as a part of the latter organization and its scientific program will be a part of the scientific program of the A.D.R.W.S.S. Members of the A.D.R.W.S.S. who have an interest in poultry diseases can apply for membership. At the organizational meeting, A. L. Kleckner, head, Department of Microbiology and Preventive Medicine, School of Veterinary Medicine, University of Georgia, Athens, was elected chairman and L. C. Grumbles, head, Department of Microbiology, School of Veterinary Medicine, Texas A. & M. College, College Station, was elected secretary.

## *Research Tools*

*Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.*

### Transistor Electrocardiograph Marker

The Taber Model 202G Electrocardiograph QRS Marker, manufactured by Taber Instrument Corp., is a completely transistorized low-frequency amplifier. It will amplify 1-millivolt heart voltages to 0.5 volt for simultaneous recording with other physiological phenomena and accentuates the QRS

lead. Heart voltages amplified with this device will produce full-scale deflections on nearly all modern cathode ray oscilloscopes.

The unit is light-weight and portable; it operates on self-contained batteries which have a 1,000-hour battery life.



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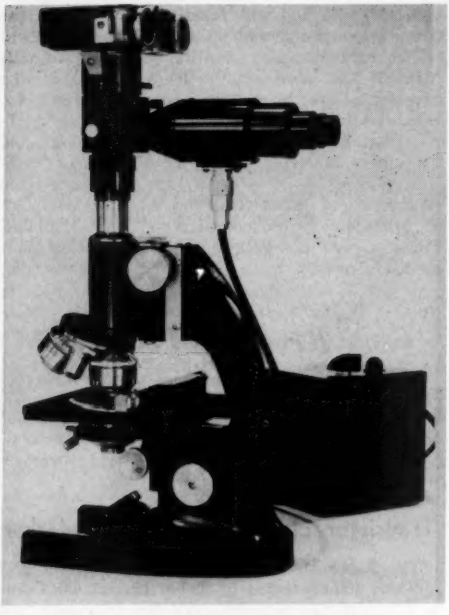
## Direct-Focusing Ultraviolet Microscope

A direct-focusing ultraviolet microscope with a 35-mm. camera for recording is available from Bausch & Lomb Optical Co.

The photomicroscope allows visual focusing of the image under ultraviolet, using the RCA "ultrascope tube." This electronic component receives



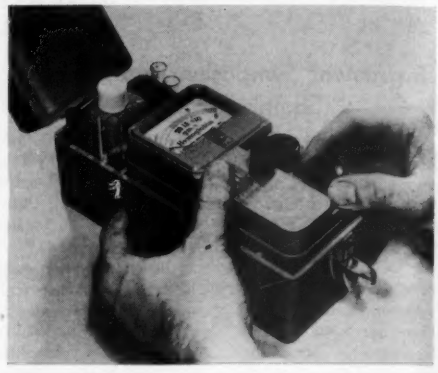
an ultraviolet image at one end and converts it to a visible image, to be viewed under magnification, at the other end. The unit is compact and portable, and is expected to have application in pathology and cytology.

The U-V Photo-Microscope includes the microscope and the ultraviolet optics and image converter; a monochromator for ultraviolet light source is available as an accessory. The image converter unit (ultrascope tube and 35-mm. camera) is available for use on existing monocular microscopes.

## Hemoglobinometer

The Coleman "25" Hemoglobinometer, manufactured by Coleman Instruments, Inc., provides exact hemoglobin measurements in approximately three minutes.

The spectrophotometer is a self-contained system that gives a direct scale reading in grams of

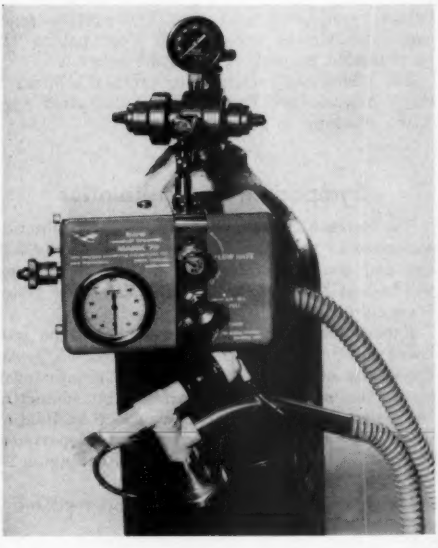


hemoglobin per 100 ml. of whole blood, eliminating preparation of slides and reference to graphs or tables.

## Demand-Breathing Respirator

An intermittent positive-pressure respirator (Bird Residual Breather Mark 7, manufactured by Instrumentation Assoc.) assists breathing under abnormal conditions.

The respirator completely fills the lungs at each inspiration, and switches on automatically when the subject fails to draw a breath. Peak inspiration, flow rate, and automatic timing (1 to 60 breaths per minute) can be adjusted to individual requirements. Air mixture and nebulization can also be controlled.



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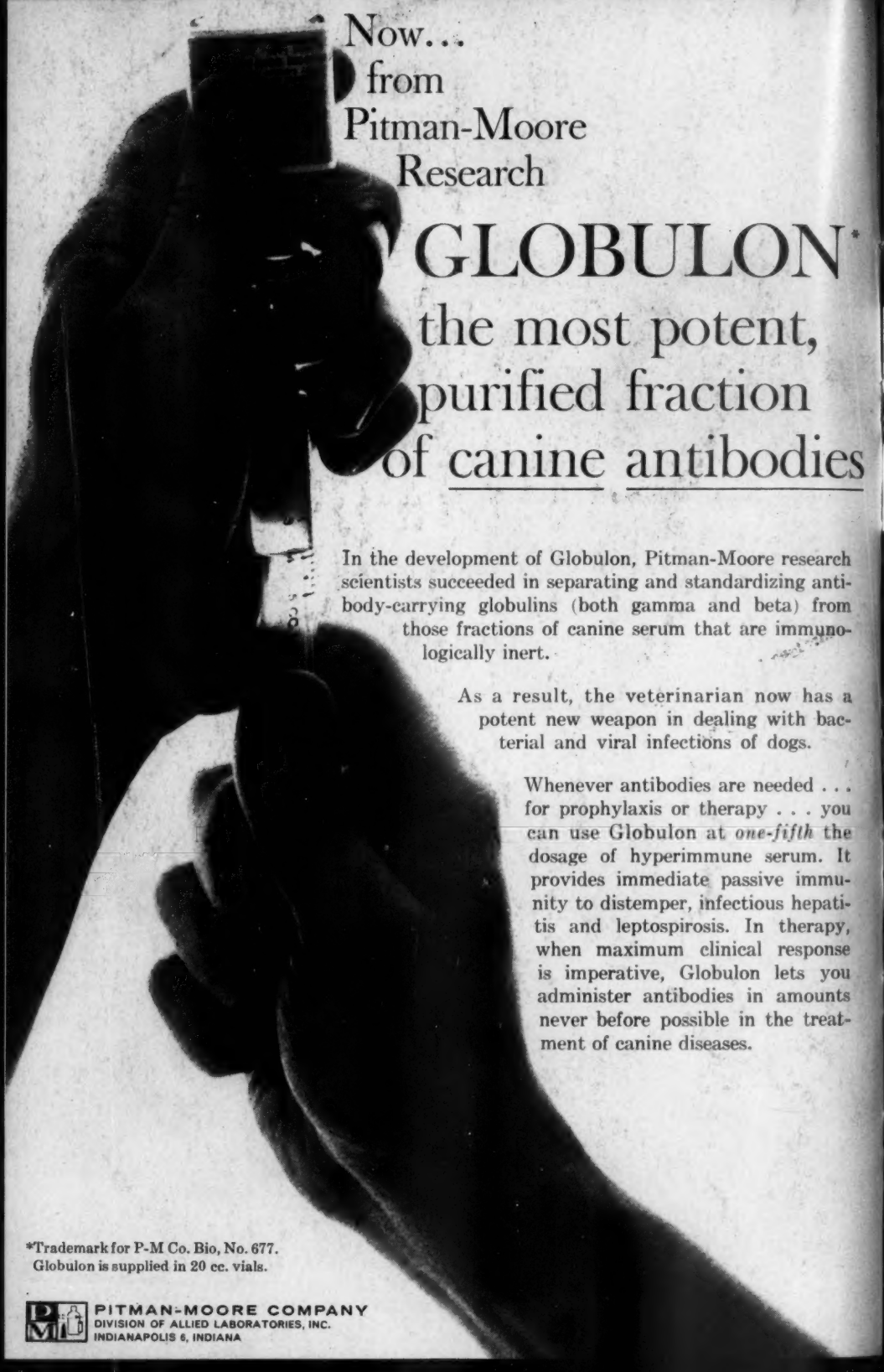

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